# Old Compounds but New Functions: Insights into Novel Modes of Action of Arabidopsis Flavonols and the Flavonol Synthase FLS1 in Modulating Cell Growth.

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## Table of contents

# Summary

Flavonoids are a large class of secondary metabolites encompassing more than 10,000 structures and are synthesized via the phenylpropanoid pathway. Flavonols, a subgroup of flavonoids, are known as negative regulators of auxin transport and are thought to have functions in signal transduction and transcriptional regulation. The activity of flavonols is modified by their glycosylation. The *rol1-2* (*repressor of lrx1*) mutant of *Arabidopsis thaliana* was isolated as a suppressor of the cell wall mutant *lrx1* (*leucine-rich repeat extensin1*) and is affected in the rhamnose synthase RHM1. The *rol1-2* mutation induces changes in the flavonol glycosylation profile, which correlates with aberrant development of the shoot including hyponastic cotyledons with brick-shaped pavement and deformed trichomes. Auxin transport is modified in the *rol1-2* mutation and is reversed to wild-type levels by blocking flavonol biosynthesis, which also suppresses the shoot phenotype of *rol1-2*.

EMS-induced suppressors of *rol1-2* led to the identification of the 7-*O*-rhamnosyltransferase locus *UGT89C1*. However, the *ugt89c1* mutations suppress the *rol1-2* phenotype not by modulating auxin transport but rather by changes in auxin metabolism. Thus, flavonols influence auxin homeostasis by targeting different auxin-related processes. Further analysis revealed that in addition to auxin, the levels of other hormones are changed in the *rol1-2* mutant and a mutation in *ugt89c1* reverts these effects of *rol1-2*.

In previous work, it was shown that flavonols might modify auxin transport by changing the activity of the antagonistic phosphatase/kinase pair PP2A/PINOID which regulate phosphorylation and hence activity and polar localization of auxin transporters. In this work, I provide *in vivo* evidence that flavonols indeed inhibit the activity of the PINOID kinase.

The flavonol synthase FLS1 shows accumulation in the nucleus, and the goal of this project was to identify the biological significance of this unexpected localization. This work reveals that in addition of producing flavonols, FLS1 affects cell growth and gene expression, and these latter functions of FLS1 dependent on the nuclear localization of the protein. The genetic separation of the different function of FLS1

shows that this enzyme has more functions than just to produce flavonols, possibly to impose information on the metabolic state of the cell on the gene regulatory network important for coordinating cell growth to prevailing conditions.

This work has investigated different aspects of developmental processes that are influenced by flavonols. It led to the identification of new functions of flavonols in addition to their known activity in regulating auxin transport and new functions of at least one enzyme involved in their synthesis. Hence, flavonols and an enzyme involved in their synthesis are more pleiotropic in their activities, adding additional layers of complexity to the mode of action by which this group of secondary metabolites influences plant growth and development.

# Zusammenfassung

Flavonoide bilden eine Gruppe pflanzlicher Sekundärmetaboliten, welche via den Phenylpropanoid Stoffwechsel synthetisiert werden. Es wurden über 10‘000 verschiedene Verbindungen nachgewiesen, welche aufgrund ihrer chemischen Eigenschaften in verschiedene Untergruppen eingeteilt werden. Die Untergruppe der Flavonole spielt eine Rolle in der negativen Regulation des Auxintransports. Zudem wird angenommen, dass Flavonole die Signaltransduktion und die Transkriptionsregulation beeinflussen. Die Aktivität von Flavonolen hängt von deren Glykosylierung ab. Die repressor of *lrx 1* (*rol1-2*) Mutante wurde als ein Suppressor der Zellwandmutante lrx1 isoliert und besitzt eine defekte Rhamose Synthase 1 *(RHM1*). Die *rol1-2* Mutation induziert Änderungen im Glykosylierungsprofil der Flavonole. Dies korreliert mit der Bildung eines Sprossphänotyps, welcher durch hyponastisch gebogene Kotyledonen mit deformierten Epidermiszellen und Trichomen beobachtbar ist. Zudem ist in der *rol1-2* Mutante der Auxintransport modifiziert. Der Shoot- und der Auxinphänotyp kann, wenn die Flavonol-Biosynthese blockiert wird, rückgängig gemacht werden.

Eine EMS Untersuchung nach *rol1-2* Suppressoren führte zur Identifikation des 7-O-Rhamnosyltransferase Lokus *UGT89C1*. Allerdings supprimieren die ugt89c1 Mutationen den rol1-2 Phänotyp nicht durch eine Modifikation des Auxintransports sondern eher durch Änderungen im Auxinmetabolismus. Demzufolge können Flavonole durch die Beeinflussung von Prozessen, welche in Zusammenhang mit Auxin stehen, auf die Auxin-Homöostase einwirken. Weitere Analysen haben gezeigt, dass in der *rol1-2* Mutante die Niveaus verschiedener Hormone verändert sind. Dies kann ebenfalls durch ugt89c1 Mutationen supprimiert werden.

In vorausgehenden Studien wurde gezeigt, dass Flavonole den Auxintransport möglicherweise durch das Verändern der Aktivität des antagonistischen Phosphatasen/Kinasen Paares PP2A/PINOID, welche die Aktivität und polare Lokalisierung von Auxintransportern regulieren, modifizieren. In dieser Arbeit

liefere ich anhand von *in vivo* Experimenten Hinweise, dass Flavonole möglicherweise die Aktivität der PINOID Kinase inhibieren.

Die FLAVONOL SYNTHASE (FLS1) ist im Zellkern lokalisiert. Daher war das Ziel dieses Projekts die biologische Signifikanz dieser unerwarteten Erkenntnis zu untersuchen. Diese Arbeit zeigt, dass FLS1, zusätzlich zur Produktion von Flavonolen, Zellwachstum und Genexpression beeinflusst. Diese neuen Funktionen sind von einer nukleären Lokalisation vom FLS1 Protein abhängig. Die genetische Trennung der verschiedenen Funktionen von FLS1 demonstriert, dass dieses Enzym nicht nur Flavonole produziert, sondern darüber hinaus dem Genregulationsnetzwerk Informationen über den Zustand des zellulären Metabolismus liefert. Dies ist bedeutend für die Koordination zwischen den vorherrschenden Bedingungen einer Zelle und Zellwachstum.

In dieser Arbeit wurde der Einfluss von Flavonolen auf verschiedene Entwicklungsprozesse analysiert. Dies führte zur Identifikation neuer Funktionen von Flavonolen zusätzlich zur bereits bekannten Rolle in der Regulation des Auxintransports. Diese Arbeit liefert Hinweise für zusätzliche Funktionen der FLAVONOL SYNTHASE (FLS1) neben der Synthese von Flavonolen. Das FLS1 Enzym wirkt auf die transkriptionelle Aktivität und Zellwachstumsprozesse ein. Folglich ist die Biosynthese der Flavonole und deren biologische Aktivität diverser als bisher angenommen. Flavonole beeinflussen ebenfalls das für ihre Biosynthese essentielle FLS1 Enzym. Dies trägt zu weiteren Komplexitätsebenen bei, wobei diese Gruppe von sekundären Metaboliten auf das Wachstum und die Entwicklung von Pflanzen einwirken.

# List of abbreviations

| | |
|---|---|
| AUX | Auxin |
| ABA | Abscisic Acid |
| CK | Cytokinins |
| JA | Jasmonates |
| BR | Brassinosteroids |
| ET | Ethylene |
| IAA | Indole-3-acetic acid |
| ACC | 1-aminocyclopropane-1-carboxylic acid |
| °C | Degree Celsius |
| MS | Murashige and Shoog |
| HPLC | High Performance Liquid Chromatograph |
| MS/MS | Tandem Mass Spectrometry |
| UPLC | Ultra Performance Liquid Chromatography. |
| EMS | Ethyl Methane Sulfonated |
| SPE | Solid phase extraction |
| qRT-PCR | Real-Time Quantitative Reverse Transcription PCR |
| Glc | Glucose |
| Rha | Rhamnose |
| ROS | Reactive Oxygen Species |
| DAPI | 6-diamidino-2-phenylindole dihydrochloride |
| DPBA | diphenylboric acid 2- aminoethyl ester |
| Asp | Aparagine |

# 3. General Introduction

### 3.1 Plant cell growth

Plant growth is achieved by controlled cell division and followed by cell expansion. In plants, compared to the most other organisms, cell expansion contributes significantly more to size yield. Entire plant organs owe their eventual size to a significant cell elongation period. In *Arabidopsis*, the cell elongation process is responsible to a great extent for the growth of hypocotyl in growing seedlings and expansion of inflorescences at the end of vegetative growth (Azpiroz, Wu, LoCascio, & Feldmann, 1998). Control of plant growth is coordinated by both external stimuli (e.g. light) and internal mechanisms (Deng, 1994).

During expansive growth, water uptake produces turgor pressure that deforms the cell wall leading to cell wall relaxation and therefore to cell expansion. Walls show both irreversible and reversible (elastic) deformation during expansive growth (Cosgrove, 2014; Cosgrove, 2016; Ortega, 1985; Ortega, 2010; Ortega and Welch, 2013). For a cell to grow, the cell surface must expand and water must enter the cell. Mostly, the proportion of cell growth is restricted by the extensibility of the cell wall while the resistance to the influx of water is relatively low. The two principal models of cell morphogenesis are diffuse growth and tip growth. In diffuse growth, expansion of the cell surface is scattered uniformly such as for cells of many algae and multicellular plants (Castle, 1955). In tip growing cells, however, such as root hairs, pollen tubes, and fungal hyphae, cells build long cylinders followed by a prolate dome where surface expansion takes place (Geitmann and Emons, 2000; Shaw et al., 2000; von Dassow et al., 2000).The expanding cell wall is made of strong, steady, and inextensible cellulose microfbrils entrenched in a hydrated polysaccharide matrix presented as pectins and hemicelluloses (Atmodjo et al., 2013; Carpita and Gibeaut, 1993; Cosgrove, 2005; Schaller et al., 2015) providing the cell wall with anisotropic mechanical properties which allow cells to extend along a favorite direction.

Different factors control cell growth. Plants are responsive to environmental cues such as water stress, light, and nutrient status, which all influence cell growth and

final cell size (Mccann and Centre, 2001). Flavonoids are involved in the key steps of cell growth and differentiation, modulating the growth of the whole plant and individual organs (DeLong, 2002).

### 3.2 The role of the flavonoids in plants

Flavonoids are secondary metabolites that modulate cell growth. This group is synthesized from phenylalanine derivatives generated via the shikimate and phenylpropanoid pathways (Tohge et al., 2013) (Figure 1), and consists of more than 10.000 structures (Agati et al., 2012). Structurally, they consist of two main groups, the 2-phenylchromans (the flavonoids, including flavanones, flavones, flavonols, flavan-3-ols, and anthocyanidins) and the 3-phenylchromans (the isoflavonoids, including isoflavones, isoflavans, and pterocarpans). Flavonols, a subgroup of flavonoids, influence a number of processes such as controlling the reactive oxygen species level (Brown et al., 1998; Peer et al., 2013) protecting from UV damages (Costa et al., 2015), signaling and transcriptional regulation (Nakabayashi et al., 2014; Peer and Murphy, 2006; Yin et al., 2012), male fertility, and defense against microbial pathogens (Dixon and Steele, 1999; Harborne and Williams, 2000; Mo et al., 1992; Winkel-Shirley, 2001) Furthermore, the flavonols in food and feed ingredients have been shown to be regulatory, protective, or pathogen-cytotoxic molecules (Butelli et al., 2008; Lin et al., 2008). Therefore, these metabolites have been the subject of intense research interest. Metabolic and nutritional engineering intends to enhance the flavonol levels in different plant species (Bovy et al., 2002; Reddy et al., 2007) such as tomato and soybean crops (Aharoni and O'Connell, 2002; Bovy et al., 2002; Yu et al., 2003).

In *Arabidopsis*, molecular and genetic characterization of *transparent testa (tt)* mutants that are compromised in the enzymes committed to the different steps in the flavonoid biosynthetic pathway has aided establishing the architecture of the pathway (Lepiniec et al., 2006) (Figure 1). These mutants display a pale-yellow seed coat because of the absence of proanthocyanidins (Debeaujon et al., 2000). Using dihydroflavonols, the FLAVONOL SYNTHASE 1 can synthesize the flavonols (Martens, 2010; Prescott et al., 2002). Although six flavonol synthase isoforms were characterized in *Arabidopsis*, only FLS1 participates in the last step of the formation

of the flavonol aglycones with significant activity (Kuhn et al., 2011; Owens et al., 2008; Stracke et al., 2009)

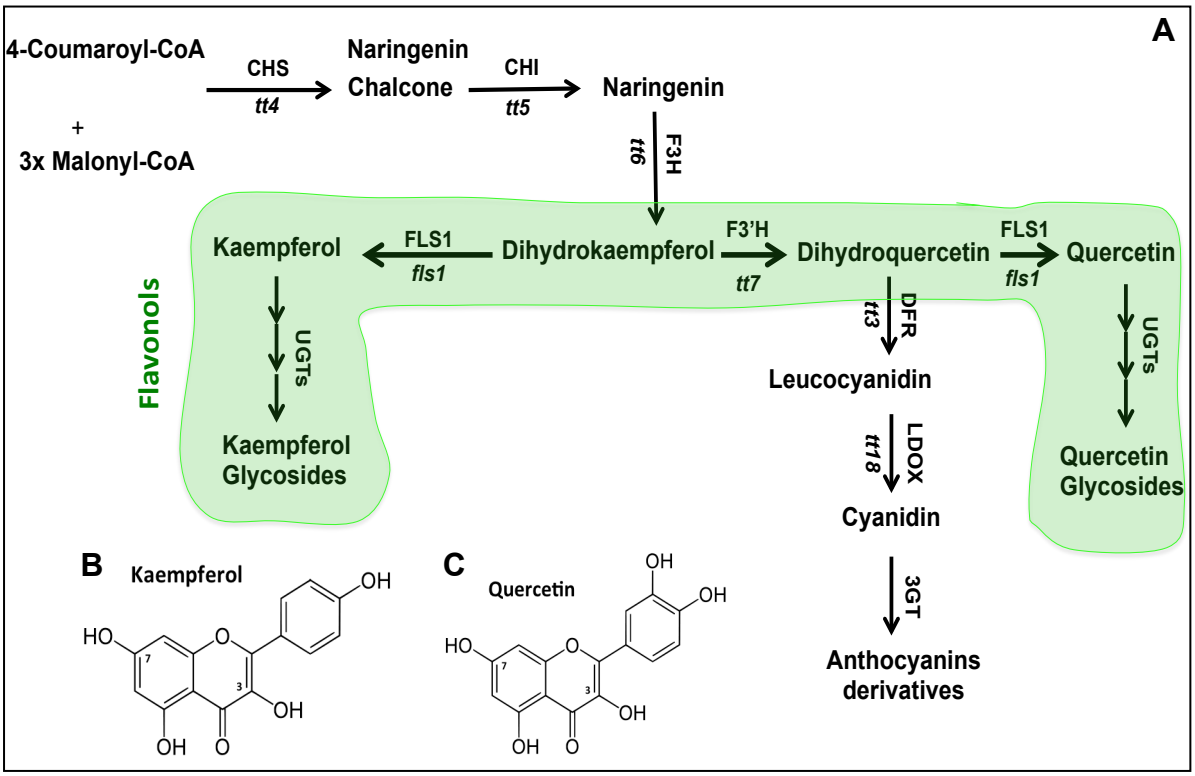


**Figure 1.** Flavonoid biosynthesis pathway. **(A)** The arrows indicate the different steps leading to flavonoid formation. Names of mutants affected in the biosynthetic process are written in italic lowercase. The first committed step in flavonoid biosynthesis is performed by CHS (CHALCONE SYNTHASE). The main flavonols Kaempferol and  are synthesized by FLS1 (FLAVONOL SYNTHASE 1). CHI, CHALCONE ISOMERASE; F3H, FLAVONOL 3-HYDROXYLASE; FLS1, FLAVONOL SYNTHASE1; F3'H, FLAVONOL 3'-HYDROXYLASE; DFR, DIHYDROFLAVONOL-4-REDUCTASE; LDOX, LEUCOANTHOCYANIDIN DIOXYGENASE; GTs, GLYCOSYLTRANSFERASES. **(B)** Kaempferol **(C)** aglycone structure and numbers indicate the main sites of glycosylation (Routaboul et al., 2006).

### 3.3 Glycosylation of flavonols

Glycosylation of flavonols is an important process conferring to these secondary metabolites their diverse chemical and physiological properties. The flavonol backbone is altered by the attachment of various functional groups and is subject to different modifications such as glycosylation, methylation, sulfation, prenylation and acylation. Glycosylation of flavonols is the main flavonol diversification in *Arabidopsis*

*thaliana.* The major chemical functions of glycosylation processes are stabilization, detoxification, solubilization, and increasing the bioavailability of these compounds (Bowles et al., 2005; Hyung Ko et al., 2006; Vogt and Jones, 2000). The flavonol aglycone backbone is subject to the attachment of rhamnose and glucose at the C3- and C7-position by the action of UDP-dependent glycosyltransferases (UGTs) (Jones et al., 2003; Tohge et al., 2005; Yin et al., 2012; Yonekura-Sakakibara et al., 2008) (Figure 1). In plants, rhamnosylated flavonols provide resistance against UV and play a role as strong insecticide agents (Lois and Buchanan, 1994; Misra et al., 2010). The other modification process that alters the properties of flavonols found in *Arabidopsis thaliana* is sulfation (Hashiguchi et al., 2014). It alters their membrane permeability through conferring a negative charge. Furthermore, sulfate binds more vigorously than to the N-1-naphthylphthalamic acid (NPA) receptor, a target of the carrier-mediated auxin efflux inhibitor NPA (Faulkner, I.J. and Rubery, 1992). Flavonols are also subject to prenylation such as in *Sophora flavescens*. This modification confers strong antifungal properties and increases the membrane permeability of flavonols (Sohn et al., 2004). In some human cancer cells, prenylation was also proposed to strengthen the binding of flavonols to ABC-transporters and thereby inhibiting pleiotropic drug resistance effects (DiPietro et al., 2002) by creating several hydrogen and ionic bonds and thus altering the 3D structure of the transporters (Wink et al., 2012). These secondary metabolites can also be interesting for agriculture or medicine as they can enhance the activity of pesticides or even reverse multidrug resistance in cells (Wink et al., 2012). However no prenylated flavonoids was detected in Arabidopsis (Sasaki et al., 2008).

### 3.4 Intracellular flavonoid transport

In many different plant species, it has been reported that flavonols are produced via a well-characterized biosynthetic pathway localized at the endoplasmic reticulum (Hrazdina, 1992; Saslowsky et al., 2005; Winkel, 2004; Winkel-Shirley, 2001). Due to their diverse functional roles in plants, flavonoids are localized in distinct cells and cellular compartments (Agati et al., 2012). Two types of transport characterize the endoplasmic reticulum-derived flavonoids: the intracellular transport toward the vacuole and the extracellular transport towards the cell wall (Burbulis and Winkel-

Shirley, 1999) (Figure 2). Vacuole transport can be performed either by membrane transporters such as ATP-binding cassette (ABC) proteins/multidrug and toxic compound extrusion (MATE) transporters or via vesicle transport (Burbulis and Winkel-Shirley, 1999; Kitamura, 2006; Zhao and Dixon, 2009) (Figure. 2). It has been speculated that the flavonoid glycosides cross the membrane of the tonoplast through the action of secondary transporters like those of the MATE family (Yazaki, 2005). However, the flavonoids conjugated to glutathione (GS) move to the vacuole especially via multidrug resistance-associated proteins (MRP)-type ABC transporters (Grotewold, 2004; Zhao and Dixon, 2009) (Figure 2).

Several studies have reported in many different plant species that vacuolar transport of flavonoids is mediated by vesicle fusing with the vacuolar membrane (Grotewold, 2004). Earlier reports have suggested that GS-X pumps associated with the ER deliver the flavonoids from the cytoplasm into the ER lumen (Grotewold, 2004). Similarly to the transport of storage proteins, vesicle-mediated transport seems not to require the Golgi apparatus (Grotewold, 2004).The vesicle-mediated transport has been particularly studied for the vacuolar accumulation of anthocyanin (Agati et al., 2012). A novel mechanism mediating the flavonoid transport such as anthocyanin has been shown. In a manner similar to microautophagy, the anthocyanin that accumulates close to the vacuole is enveloped. The single membrane of the tonoplast surrounds the enveloped anthocyanin during this process and the compounds become free in the vacuolar lumen (Chanoca et al., 2015) (Figure. 2). These anthocyanin vacuolar inclusions have been found in the case of grape berries and *Arabidopsis thaliana* (Agati et al., 2012; Chanoca et al., 2015; Conn et al., 2010; Gomez et al., 2011; Petrussa et al., 2013; Zhang et al., 2007).

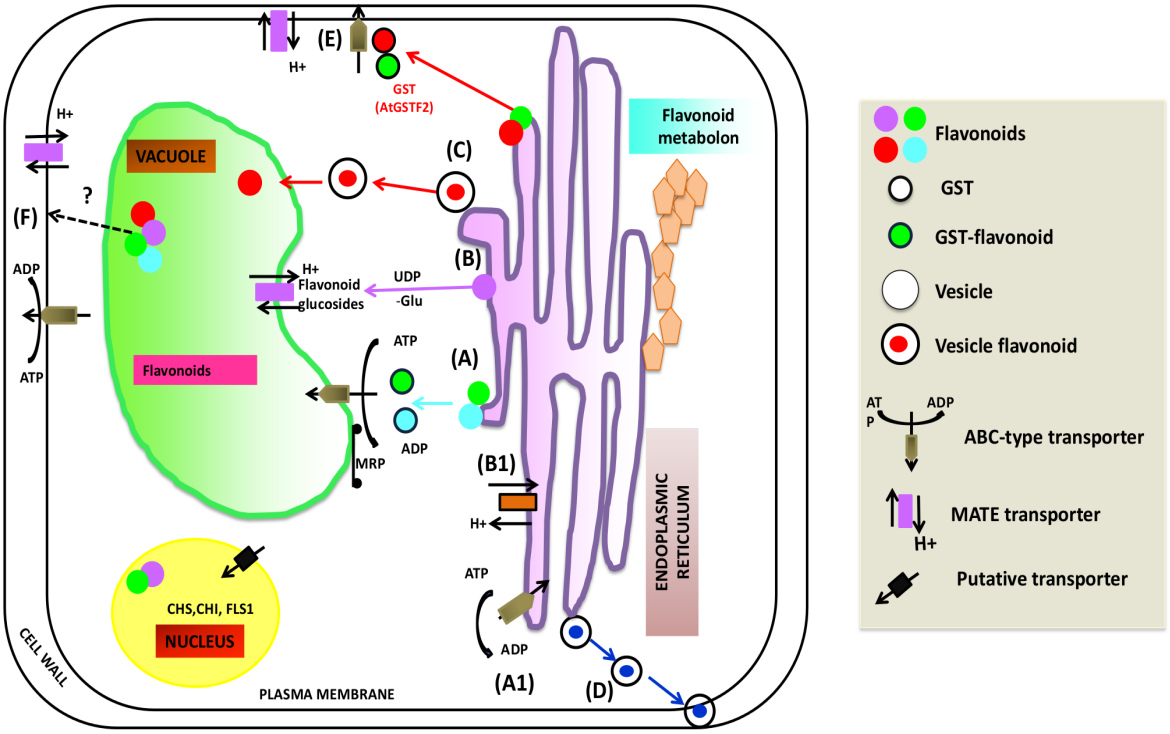


**Figure 2.** Flavonoid biosynthesis pathway and transport mechanisms of Endoplasmic Reticulum (ER)- derived flavonoids to the vacuole and the cell wall. **(A):** Flavonoid transport mediated by ABC-type transporters. (**B):** Flavonoid cross the tonoplast using an H+-energized mechanism. **(C):** Vesicle-mediated transport of flavonoids (Anthocyanin). **(A1):** ABC-type transporters. **(B1):** Multidrug and toxic ions extrusion (MATE) transporters. **(D):** Flavonoids accumulate in the cell wall through vesicle-mediated transporters. **(E):** Using GS-flavonoid complexes, flavonoids are extruded from the cell and accumulate in the cell wall. **(F):** Release of flavonoids from the vacuole that might cross the plasma membrane using both ABC-and MATE-type proteins. Whether flavonoids are transported to the nucleus and the chloroplast is unknown. Adopted from Agati *et al.* (2012).

### 3.5 Differential localization of flavonoid biosynthesis enzymes

In *Arabidopsis thaliana* as well as numerous other species such as *Tsuga canadensis*, *Brassica napus, Flaveria chloraefolia, Taxus baccata, and Picea abies,* the nuclear accumulation of flavonoids was described (Agati et al., 2012; Buer and Muday, 2004; Feucht et al., 2004; Grandmaison and Ibrahim, 1996; Hutzler1 et al., 1998; Kuras, M, Wronka, M., Linch, J.M. & Zobel, 1999; Peer, 2001). However, the functional role of nuclear flavonoids in plant cells is not fully understood. It has been speculated that these compounds protect DNA from UV and oxidative damages

(Feucht et al., 2004). Moreover, a transcriptional regulation of genes was attributed to these metabolic compounds, especially, genes linked to growth and development including auxin transport-related genes. The mechanism of this regulatory function is still unclear (Buer and Muday, 2004; Grandmaison and Ibrahim, 1996; Kuras, M, Wronka, M., Linch, J.M. & Zobel, 1999). In mammalian cells, the action of polyphenols as epigenetic modifiers is especially described in cancer-related models. Treatment of various cancer cell lines with flavonols or isoflavones reduced the methylation status of the promoter and increased mRNA expression of numerous tumor suppressor genes (Fang et al., 2003). The discovery that enzymes involved in key steps of flavonol biosynthesis can localize in the nucleus e.g. CHALCONE SYNTHASE, CHALCONE ISOMERASE and FLAVONOL SYNTHASE 1, as shown for *Arabidopsis* is intriguing (Kuhn et al., 2011; Saslowsky et al., 2005) (Figure, 2). It supports the suggested function for nuclear flavonoids in the regulation of the gene expression required for growth and development (Saslowsky et al., 2005), but still does not explain the biological significance of these enzymes in the nucleus since flavonoids biosynthesis is thought to take place at the ER.

### 3.6 Transcriptional regulation of flavonoid biosynthesis genes

The transcriptional regulation of the flavonoid biosynthesis pathway is well described. The *Arabidopsis thaliana* genes such as *TESTA GLABRA 1, TRANSPARENT TESTA 2 (TT2)* and *TRANSPARENT TESTA 8 (TT8)* encode *WD40*, *MYB,* and *bHLH* transcription factors respectively. They form a transcriptional complex that modulates gene expression of e.g. the *LEUCOANTHOCYANIDIN DIOXYGENASE (LDOX), ANTHOCYANIDIN REDUCTASE (ANR)* and *DIHYDROFLAVONOL 4-REDUCTASE* (DFR) (Dubos et al., 2008; Fang et al., 2003). Both post-transcriptional modification and transcriptional controls have been implicated in this regulation. Several R2R3-MYB-related and bZIP-type transcription factors regulating flavonol biosynthesis have been described, which are themselves also subject to transcriptional control (Dubos et al., 2008; Lillo et al., 2008; Olsen et al., 2009; Stracke et al., 2007; Stracke et al., 2010). The three flavonol-specific regulators MYB11, MYB12 and MYB111 induce expression of the genes encoding the biosynthetic enzyme CHALCONE SYNTHASE (CHS), CHALCONE

ISOMERASE (CHI), FLAVANONE 3-HYDROXYLASE (F3H) and FLAVONOL SYNTHASE1 (FLS1) (Mehrtens, 2005; Stracke et al., 2007). These four genes are co-regulated in *Arabidopsis thaliana* (Hartmann et al., 2005) and are mandatory for the formation of the basic flavonol aglycone from p-coumaroyl-CoA (Stracke et al., 2010) (Figure 1). In growing seedlings and because of their differential spatial activities, MYB12 and MYB111 act in an additive manner. MYB12 induces flavonol biosynthesis in the root whereas MYB111 primarily regulates flavonol biosynthesis in cotyledons (Stracke et al., 2007). However, in the seedling, MYB11 seems to have only a minor role in the control of flavonol biosynthesis.

### 3.7 The role of auxin in plant growth

Auxin moves from cell to cell with a polarity and is distributed asymmetrically throughout the plant body. In a basipetal manner, auxin moves from the shoot apex to the basal region. In roots, auxin moves in both acropetal manner through the central tissues and basipetal manner through the epidermis (Friml et al., 2002a; Friml et al., 2002b; Friml et al., 2003; Lomax et al., 1995). This vectorial auxin flow is known to be involved in diverse plant growth and developmental processes, such as vascular development, gravitropism, phototropism, apical dominance, and pattern formation during embryogenesis (Kitamura, 2006; Lomax et al., 1995; Morris et al., 2004).

In *Arabidopsis thaliana,* polar transport of auxin is essentially accomplished by three families of plasma membrane-associated transporter proteins, namely PIN-FORMED (PIN) efflux carriers (Petrášek et al., 2006). AUXIN RESISTANT1/LIKE AUX1 influx carriers (Bennett et al., 1996; Swarup et al., 2008; Yang et al., 2006) and P-GLYCOPROTEIN ATP-Binding Cassette ABC transporters (Geisler et al., 2005; Noh et al., 2001). Among these transporters, PIN auxin efflux carriers play pivotal roles in directing intercellular auxin flow and mediating the auxin-regulated developmental processes through their asymmetric and polar localization at the plasma membrane (Wisniewska, 2006). For example, localization of PIN1 at the apical ends of cells in the stele of the root is shown to promote clear auxin shift toward the root tip (Blilou et al., 2005). Laterally symmetric PIN3 in the columella cells of a vertically growing root mediates a homogeneous centrifugal flow of auxin toward the flanks. (Friml and

Palme, 2002). The basipetal auxin transport is facilitated by one of the efflux carriers namely PIN2 (Chen et al., 1998; Müller et al., 1998).

### 3.8 Modulation of auxin homeostasis in plants

Protein phosphorylation and dephosphorylation processes are involved in the modulation of auxin transport (Lee and Cho, 2006). In *Nicotiana tabacum* suspension cells, kinase inhibitors e.g. K252a and staurosporine and phosphatase inhibitors e.g Cantharidin have been shown to inhibit efflux/ influs of auxin (Delbarre et al., 1998). *In Arabidopsis*, antagonistic effects of the protein serine/threonine (Ser/Thr) kinase PINOID (PID) and the phosphatase PROTEIN PHOSPHATASE 2A (PP2A) influence polar PIN localization (Michniewicz et al., 2007; Muday and DeLong, 2001; Rashotte et al., 2001; Xi et al., 2016). Using the root hair cells of *Arabidopsis thaliana* as a system and the tobacco (*Nicotiana tabacum)* suspension cell system, the protein kinase PINOID (PID) was shown to positively regulate auxin efflux (Benjamins et al., 2001; Christensen et al., 2000; Friml, 2004; Lee and Cho, 2006). A reduction in PP2A activity and PID overexpression increase PIN phosphorylation, leading to a basal-to-apical shift in PIN polarity and consequently a root agravitropic phenotype (Friml, 2004; Michniewicz et al., 2007; Rashotte et al., 2001; Xi et al., 2016; Zhou et al., 2004).

Flavonoids are known as negative regulators of polar auxin transport (Brown et al., 2001; Buer and Muday, 2004; Murphy et al., 2000; Peer, 2001; Peer and Murphy, 2006; Peer and Murphy, 2007; Peer et al., 2004; Peer et al., 2011). The two endogenous flavonols, Kaempferol and,are able of greatly inhibit auxin efflux carriers (Brown et al., 2001; Murphy et al., 2000). Furthermore, flavonoid deficient transparent testa 4 *(tt4)* mutants lacking flavonoid biosynthesis have been shown to exhibit elevated root basipetal auxin transport compared with the wild type, consistent with the absence of a negative auxin transport regulator (Brown et al., 2001; Buer and Muday, 2004). The localization of PIN auxin-carriers, important for the reflux, is altered in *tt4* (Peer et al., 2004). Other studies have suggested flavonols as modulators of another class of auxin efflux transporters such as the B group ATP-

binding cassette transporter superfamily (ABCB) (Blakeslee et al., 2007; Brown et al., 2001; Geisler et al., 2005). The Ser/Thr protein kinase PINOID (PID) has been shown to regulate the activity of auxin transport (Kleine-Vehn et al., 2009). It targets the PIN proteins localization and ABCB transporter activity (Aryal et al., 2015; Friml et al., 2003; Michniewicz et al., 2007; Mravec et al., 2008; Petrášek et al., 2006). The PIN phosphorylation status is controlled by the opposite activity of PID kinase and the Ser/Thr phosphatases type 2A (PP2A) (Huang et al., 2010; Michniewicz et al., 2007). Interestingly, flavonols were shown to inhibit the PID activity *in vivo* (Henrichs et al., 2012).

### 3.9 Mutual influences of phytohormones in different biological processes

Plant growth and developmental processes have shown to be regulated by both extrinsic cues and internal growth regulators, such as hormones (Santner and Estelle, 2010). Increasing evidence proposes that environmental signals target the biosynthesis or perception of hormones, which therefore not only coordinate intrinsic developmental programs, but also convey environmental inputs (Santner et al., 2009). Different phytohormones, including auxin, interact and affect common processes, in a way that the output of this mutual synergy depends on specific hormone combinations rather than on the individual action of each of them (Weiss and Ori, 2007). Plant hormones consist of small molecules derived from different metabolic pathways and are crucial modulators of plant development. They operate at nanomolar concentrations and the five types of the classical hormones, namely, are auxins, cytokinins, abscisic acid, gibberellins, and ethylene. Other types of hormones were recognized as new families of plant hormones such as jasmonates, strigolactones, salicylates, brassinosteroids, polyamines, and some peptides (Munné-Bosch and Müller, 2013). Hormones construct a signaling network and tightly modulate various signaling and metabolic processes that are essential for plant development and plant responses to biotic and abiotic stresses. It is clearly evident that physiological processes are regulated in a complicated manner by the crosstalk of various hormones.

The crosstalk of the three hormones auxin, ethylene, and cytokinin in *Arabidopsis* was investigated in terms of root development. These activities display either

synergistic or antagonistic interactions depending on the cellular context (Liu et al., 2013). Auxin inhibits the activation of axillary buds, and hence shoots branching, while cytokinin has the opposite effect (Mller and Leyser, 2011). On the root level, this antagonistic effect is essential for specifying the embryonic root stem cell niche during *Arabidopsis thaliana* embryogenesis (Kushwah et al., 2011). A network of hormones and genes coordinates plant growth by closely controlling the activity of hormone signaling pathways. For instance, Ethylene signalling modulates *PIN1 and PIN2* genes whereas the effect of cytokinin involves an auto-regulatory role in addition to the influence on the levels of auxin and ethylene (Liu et al., 2013; Ruzicka et al., 2007; Ruzicka et al., 2009). Another aspect of hormone crosstalk is between gibberellin and the phytohormones such abscisic acid, auxin, ethylene and cytokinin. Gibberellin and abscisic acid play antagonistic roles in the regulation of a plethora of developmental processes. While gibberellin is known to promote germination, growth, and flowering, abscisic acid prevents these processes (Weiss and Ori, 2007). Furthermore, this antagonistic relationship between these two hormones is seen in the transition from embryogenesis to seed germination (Razem et al., 2006). In contrast, the action of Gibberellin and auxin is synergistic in terms of the regulation of cell expansion and tissue differentiation. For instance, *In Arabidopsis thaliana*, gibberellin stimulation of root elongation has been shown to require auxin (Fu and Harberd, 2003). The interaction between Gibberellin and Ethylene is quite complex, as both antagonistic and synergistic effects have been determined (Saibo et al., 2003). For example, gibberellin enhances seedling root elongation in *Arabidopsis thaliana*, whereas this response is inhibited by ethylene (Achard et al., 2003). Cytokinin and gibberellin act in an antagonistic way and effect various growth processes such as elongation of shoot and root, shoot regeneration in culture, cell differentiation, and meristem activity (Greenboim-Wainberg et al., 2005; Jasinski et al., 2005). In summary, plant hormones interact in complicated networks to balance the response to developmental and environmental signals.

### 3.10 Hormonal crosstalk in stomatal aperture

The guard cells, two very distinct cells, that encircle the stomatal pore, are capable to incorporate environmental signals and endogenous hormonal stimuli in order to

control the stomatal aperture. They restrict water loss by regulating the transpiration and exchange rate of mainly $CO_2$ and $O_2$, which are essential for photosynthesis. Guard cells react to several determinants, which are translated into the complicated network of signaling pathways that control stomatal actions. The perception of an abiotic stress provokes the activation of signal transduction cascades that interfere with/or are activated by phytohormones. Among these, abscisic acid is the best-known hormone that induces stomatal closure (Schroeder et al., 2001) (Figure 3). Interestingly, other plant hormones, namely jasmonic acid, brassinosteroids, cytokinins, or ethylene are also implicated in the response of stomata to stresses (Daszkowska-Golec and Szarejko, 2013; Huang et al., 2008; Nemhauser et al., 2006; Watkins et al., 2014) (Figure 3). The alteration of gene expression in response to ABA affects genes that are connected to cytokinins, ethylene, and auxin signaling (Daszkowska-Golec and Szarejko, 2013). The intricacy of this response is essentially dependent on the primary threshold of stress and the particular plant's stress history. Mainly, abscisic acid and jasmonic acid are positive regulators of stomatal closure; in contrast, auxin and cytokinins are positive regulators of stomatal opening and these latter affect ethylene biosynthesis. However ethylene has an antagonistic effect on ABA-induced stomatal closure (Tanaka et al., 2006). Furthermore, the mechanism of action of ethylene is puzzling because it acts as either a positive or negative regulator, according to the tissue and conditions (Huang et al., 2008; Nemhauser et al., 2006). Nitric oxide and jasmonic acid are also involved in the stimulation of the stomatal closure and seem to interfere with ABA as a part of the drought response (Figure 3).

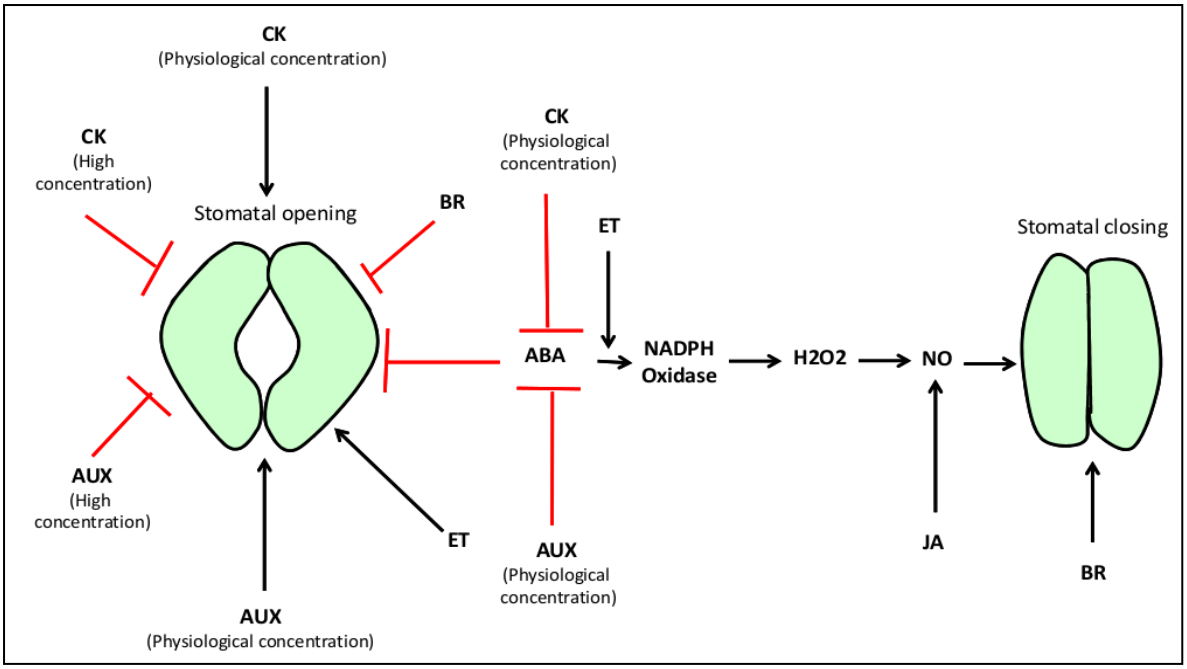


**Figure 3.** Hormonal crosstalk in the regulation of stomatal closure and opening during water stress. The regulation of stomatal opening and closure is not only regulated by ABA, whose role is dominant, but also by other phytohormones. Jasmonates (JA) and Brassinosteroids (BR) induce stomatal closure and inhibit stomatal opening under drought conditions, whereas the role of other hormones is ambiguous. Cytokinins (CK) and Auxins (AUX) in low physiological concentrations promote stomatal opening while in high concentrations, they are able to inhibit this process. The role of Ethylene (ET) is the most curious. It can stimulate the closing and opening of the stomata. Adopted from Daszkowska-Golec and Szarejko (2013).

### 3.11 Characterization of *rol1-2* a repressor of *lrx1*

The *rol1-2* mutant, identified as a repressor of the LRR-extensin mutant *lrx1* that causes a root hair phenotype (Baumberger et al., 2001), is altered in one of the tree rhamnose biosynthesis genes (*RHM*) that is *RHM1*. RHMs catalyze the conversion of UDP-Glc to UDP-Rha (Diet et al., 2006). The *rol1-2* mutant exhibits modifications in the Rha-rich cell wall component pectin (Diet et al., 2006), and shows changes in the flavonol glycosylation profile, mainly a reduction in the rhamnosylation level compared to the wild type (Ringli et al., 2008). This emphasizes the importance of *RHM1*, which is co-expressed with genes important for flavonol biosynthesis (Yonekura-Sakakibara et al., 2008) and for flavonol glycosylation. Short roots and root hairs, hyponastic cotyledons, disturbed adaxial pavement cells of cotyledons consisting of brick-like cell shape, and aberrant trichomes on the first rosette leaves

are the characteristics of the *rol1-2* mutant. In contrast, normal trichomes, and epinastic cotyledons having the puzzle-shaped jigsaw-like pavement cells distinguish the wild type (Diet et al., 2006; Kuhn et al., 2011; Ringli et al., 2008).

The shoot phenotype of *rol1-2* is related to the altered flavonol composition in *rol1-2.* In contrast, the short root and root hair phenotype of the *rol1-2* mutant seems to be induced by the changes in pectin structures (Diet et al., 2006). *fls1-3* was isolated as a suppressor of *rol1-2* (Kuhn et al., 2011). The *rol1-2 fls1-3* double mutant shows the re-conversion of the *rol1-2* shoot phenotype to a wild type-like phenotype because of blocking the flavonol biosynthetic pathway (Kuhn et al., 2011). Furthermore, the auxin transport is altered in rol1-2 but reverted to wild-type level in the *rol1-2 fls1-3* (Kuhn et al., 2011). The root phenotype in *rol1-2* plants is not strongly affected by *fls1-3* mutation. It appears that flavonols do not affect the root cells important for the *ro1-2* phenotype. For example, they accumulate only poorly in root hairs based on the low expression of *FLS1*.

**Aims of the thesis**

The *rol1-2* mutant is a very useful tool to identify physiological processes that are regulated by flavonols. It led to the identification of flavonols as regulators of plant development and modulators of auxin transport. Here in this study, I aimed to understand the different modes of action of flavonols to modulate plant growth and development by looking at the impact of the changes in flavonols glycosylation profile on auxin homeostasis and to assess the *in vivo* effect of flavonols on PID activity during the gravitropic and phototropic response. Finally, this project revealed a new finding, which demonstrates that FLS1 not only acts as the enzyme for flavonol biosynthesis but also has additional functions in the nucleus and impacts gene expression and cell growth.

# 4. Flavonol-induced changes in PIN2 polarity and auxin transport in the *Arabidopsis thaliana rol1-2* mutant require phosphatase activity

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My contribution to this work was the *in vivo* analysis of the negative regulation of PID kinase by the flavonols in the Arabidopsis root.

### 4.1 Abstract

The phytohormone auxin is a major determinant and regulatory component important for plant development. Auxin transport between cells is mediated by a complex system of transporters such as PIN and ABCB proteins, and their activity is influenced by phosphatases and kinases. Flavonoids are secondary metabolites that modify auxin accumulation and transport activity. The Arabidopsis *thaliana rol1-2* mutant is affected in the accumulation of flavonols, a subgroup of flavonoids, and is characterized by defects in cell growth and altered auxin transport activity. A new mutation in *ROOTS CURL IN NPA 1* (*RCN1*), encoding a regulatory subunit of the phosphatase PP2A, was identified as a suppressor of *rol1-2*. *rol1-2 rcn1-3* double mutants show alleviation of the growth defects and a reversion of the auxin transport activity to wild type-like levels. PIN protein localization revealed a basal-to-apical shift of PIN2 in cortical cells of the *rol1-2* mutant, which is reversed in *rol1-2 rcn1-3* to basal localization. *In vivo* analysis of PINOID action, a kinase known to influence PIN protein localization in a PP2A-antagonistic manner, revealed a negative impact of flavonols on PINOID activity. Together, these data indicate that flavonols affect auxin transport activity by modifying the antagonistic kinase/phosphatase equilibrium.



Key words: *Arabidopsis thaliana*, *rol1-2*, flavonols, auxin, auxin transport, PIN2, RCN1, flavonoids

## 4.2 Introduction

Plant growth and development is influenced and regulated by a network of phytohormones. Among those, auxin is involved in a large number of processes. An important characteristic of auxin is the unequal distribution between cells, which is a prerequisite for cellular differentiation, signaling, and cell division. This unequal distribution of auxin is induced by polar auxin transport (PAT) between cells and involves the action of auxin transporters (Adamowski and Friml, 2015; Pencik *et al.,* 2013). PAT is mediated by a number of transporters of the PIN and ABCB class of proteins. ABCB's are mainly apolarly localized and are involved in the long-range auxin transport (Bailly et al., 2008; Blakeslee et al., 2007; Bouchard et al., 2006; Geisler et al., 2005). PINs often show polar localization, export auxin, and are important for the reflux observed in the root apex resulting in a vectorial auxin stream (Friml et al., 2003; Mravec et al., 2008; Petrasek et al., 2006; Wisniewska et al., 2006). *pin* loss of function mutants result in strong phenotypes, underlining their importance for auxin distribution and plant development (Blilou et al., 2005; Vieten et al., 2007). Auxin transport activity is regulated also on the post-translational level by the phosphorylation status of transport proteins. The PINOID (PID) kinase is a central component in this process, which regulates organ development by enhancing PAT and modifying responses to auxin. Consequently, a *pid* mutant shows developmental defects (Benjamins et al., 2001; Bennett et al., 1995; Christensen et al., 2000). PID kinase activity influences the activity of ABCB1 by phosphorylating the regulatory linker region of this protein (Henrichs et al., 2012). PIN-dependent auxin transport was shown to be regulated by (de-) phosphorylation activity which influences the position as well as the activity of these transporters (Friml et al., 2004; Garbers et al., 1996; Shin et al., 2005; Zourelidou et al., 2014). The phosphorylation status of PIN proteins in the central hydrophilic loop is determined by the antagonistic activity of the PID kinase and the phosphatase PP2A (Huang et al., 2010; Michniewicz et al., 2007). As a consequence, auxin transport in roots of *pid* mutants is reduced (Sukumar et al., 2009) while it is increased in the *pp2aa1* mutant *rcn1* (*roots curl in NPA*) (Muday et al., 2006; Rashotte et al., 2001). Mutations in *rcn1*, a regulatory A subunit of PP2A (PP2AA1) or the other two regulatory A subunits PP2AA2 and PP2AA3 influence phosphorylation of PIN proteins, auxin transport, and auxin-related processes (Michniewicz et al., 2007).

Flavonoids are a large group of plant secondary metabolites produced via the phenylpropanoid pathway that serve diverse functions in UV protection, pathogen defense, plant-microbe communication, regulation of reactive oxygen species, and auxin transport (Taylor and Grotewold, 2005). Interfering with flavonoid biosynthesis results in defects in root hair and pollen tube growth in petunia and maize (Mo et al., 1992; Taylor and Grotewold, 2005). Flavonols are a subgroup of flavonoids produced by the FLAVONOL SYNTHASE 1, FLS1, and in *Arabidopsis thaliana* largely consist of Kaempferol and that are glycosylated predominantly by Glc and Rha at the C3 and C7 position resulting in a large variety of glycosidic forms (Lepiniec et al., 2006; Veit and Pauli, 1999). A number of *transparent testa* (*tt*) mutants were identified based on the pale color of the seed coat due to the absence of proanthocyanidins, a final product of the phenylpropanoid pathway (Koornneef, 1990; Shirley et al., 1995). Interfering with flavonoid biosynthesis in Arabidopsis by mutating *tt4*, encoding CHALCONE SYNTHASE committed to the initial step in flavonoid biosynthesis, results in altered cycling of PIN1 and increased auxin transport. By contrast, the -deficient and Kaempferol-overaccumulating *tt7* mutant shows inhibited auxin transport (Buer and Muday, 2004; Peer et al., 2004). Flavonols have been shown to compete with the auxin transport inhibitor 1-*N*-naphtylphtalamic acid (Jacobs and Rubery, 1988), can inhibit PID kinase activity (Henrichs et al., 2012), and redirect PIN-mediated auxin fluxes (Peer et al., 2004; Santelia et al., 2008).

The *rol1-2* mutant is affected in rhamnose biosynthesis due to a mutation in the *RHAMNOSE SYNTHASE 1* (*RHM1*), resulting in changes in rhamnose-containing pectin and a modified flavonol glycosylation profile with more glucosylated and less rhamnosylated flavonols compared to the wild type. Compared to the wild type, *rol1-2* seedlings develop shorter roots and root hairs, hyponastic instead of epinastic cotyledons, brick-shaped instead of jigsaw-puzzle shaped pavement cells in cotyledons, and deformed trichomes on the first rosette leaves (Diet et al., 2006; Ringli et al., 2008). Blocking flavonol biosynthesis in the *rol1-2* mutant background by mutations in genes encoding *FLS1* or enzymes in earlier steps of flavonol biosynthesis suppresses the *rol1-2* shoot phenotype, indicating that flavonols present in *rol1-2* interfere particularly with proper shoot development. The root phenotype of the *rol1-2* mutant, in contrast, is only slightly suppressed in the

absence of flavonoids, which does not exclude a function of flavonols in root development but suggests that the pectin-induced short-root phenotype of *rol1-2* seedlings is epistatic over the alterations induced by the flavonols (Kuhn et al., 2011; Ringli et al., 2008). *rol1-2* mutant shoots show altered auxin signaling and transport activity, effects that are alleviated by preventing flavonol biosynthesis by mutations in *tt4*, *tt6*, *fls1*, or *myb111*, the latter being a transcriptional regulator of flavonol accumulation (Kuhn *et al.,* 2011; Ringli et al., 2008; Stracke et al., 2007). Together, these findings suggest that flavonols present in the *rol1-2* mutant induce alterations in plant development by modifying cellular processes such as auxin transport. Recently, mutations in the flavonol 7-rhamnosyltransferase gene *UGT89C1* were found to suppress *rol1-2*. This suppression, however, is not caused by alterations in the transport but in the levels of auxin metabolites (Kuhn et al., 2016), indicating that flavonols can also influence auxin turnover.

Here, we describe the identification of a suppressor of *rol1-2* that does not influence flavonol accumulation *per se*. The identified *rcn1* mutation alleviates the modified auxin transport activity in *rol1-2*. PIN localization analysis revealed that flavonols in *rol1-2* cause a partial shift in PIN2 polarity and this requires the activity of PP2A. Our data indicate that the flavonols in *rol1-2* mutant negatively influence the PID kinase and reducing the activity of the antagonistic PP2A by means of the *rcn1-3* mutation alleviates the effect of flavonols.

## 4.3 Materials and Methods

### Plant material and growth conditions

All plant lines are in the Columbia (Col_0) genetic background. The *rol1-2* and *fls1-3* mutants and the molecular markers for the mutations are described elsewhere (Diet et al., 2006; Kuhn et al., 2011). The polymorphism of the *rcn1-3* allele (G to A substitution at position 2488 relative to the start codon of the genomic sequence) is detected by PCR with the primers *N1759_F* CAGAGGAGTTTGGTCCTCCATG (positions underlined are mutated compared to the wild-type RCN1 sequence) and *N1759_R* CTCAATATTTGCAGCTTTAGTG, followed by digestion with *Nco* I, which only cuts the wild-type sequence.

The identified *rol1-2 rcn1-3* double mutant was back-crossed several times to *rol1-2* and a 1:3 ratio of suppressed versus *rol1-2* phenotype was observed, confirming the recessive nature of *rcn1-3*.

Seeds were surface sterilized (1% sodium hypochlorite, 0.03% TritonX-100) and stratified 2-4 days at 4°C. Seeds were plated on 1/2 Murashige and Skoog medium (0.6% phytagel (Sigma), 2% sucrose, 100µg/mL myo-inositol) and grown in growth chambers with 16 h light, 8 h dark cycles at 22°C.

**DNA constructs and plant transformation**

For the *RCN1* complementation construct, the *RCN1* genomic clone was PCR-amplified with the primers *PP2A_CF* CTATAAGACTTGTGATATCAATTG and *PP2A_CR* CTCTTGGAAAATAGGAGATATAAC, encompassing 1.5 kb promoter, 3.2 kb coding sequence (CDS), and 0.5 kb terminator sequence. The resulting fragment (*RCN1:RCN1*) was cloned into *pGEM-T easy* (Promega) for sequencing. For the GFP fusion construct, a *Kpn* I was introduced into *RCN1:RCN1* clone by PCR 3‟ of the ATG (N-terminal fusion). PCR was performed with the primers *PP2A_NGFP_F* GGTACCGCTATGGTAGATGAACCG and *PP2A_NGFP_R* GGTACCCATCTTATGTGAAAGTTCG . A previously produced *GFP* construct flanked by *Kpn* I sites was cloned into these *Kpn* I sites, resulting in *RCN1:GFP-RCN1*. These constructs were cloned into the binary vector *pBART27* (Gleave, 1992; Stintzi and Browse, 2000) by *Not* I.

For the *FLS1:PID* construct, the *FLS1* promoter was amplified with the primers *FLS1_F3_PC* AATTTCTACTGAATTCGACAGAG and FLS1_Prom_R GGATCCTATTTTTTTTGGTAGTTTGCGTTGC, the FLS1 terminator with the primers *FSL1_Term_F* CTCGAGTGAGAAAAATCAATACGAGAAGAATA and *FLS1_R3C_R* TAATAGCGAATGTGTCGGTTTG, and the PID genomic coding sequence with the primers *PID_FLS_F* GGATCCATGTTACGAGAATCAGAC and *PID_FLS_R* CTCGAGAAAGTAATCGAACGCCGC. The PCR fragments were cloned into *pGEM-T easy* (Promega) for sequencing and correct clones were used to fuse the three fragments in *pGEM-T easy* via the *Bam* HI site at the *FLS1* promoter-*PID* border, and the *Xho* I site at the *PID -FLS1* terminator border. A correct construct was cloned into the binary vector *pART27* (Gleave, 1992) by *Not* I.

Plants transformed with the binary vectors *pBART27* or *pART27* (Gleave, 1992; Stintzi and Browse, 2000) were selected on MS medium containing 100 µg/mL Ticarcillin and either 10 µg/mL basta or 50 µg/mL kanamycin, respectively. T2 lines of transgenic lines were grown on basta or kanamycin-containing plates and lines segregating 3:1 for resistance indicating T-DNA integration at one genetic locus were subsequently used for analysis. Plants containing the *FLS1:PID* transgene were crossed into to introduce the transgene in the different genetic backgrounds.

**Microscopic analyses**

Light microscopic observations were made using a Leica DM6000 stereomicroscope. Imprinting of pavement cells was done as published (Horiguchi et al., 2006) and observed with a Zeiss AX10 microscope.

**Root length and gravitropism measurements**

For root length quantification, seedlings were grown for six days on MS medium in a vertical orientation. Agar plates were subsequently scanned and root length was determined using ImageJ. For gravitropic response analysis, plants were grown for six days on MS plates, laid out straight on fresh MS plates and incubated in the dark. After 24 hrs, the plates were turned by 90$^{o}$ and then scanned after 2, 4, and 8 hrs. Angles were determined using ImageJ.

For determining the vertical growth index, plants were grown for six days on MS plates, scanned, and then analyzed by ImageJ.

**PIN protein localization**

Automated whole-mount protein immunolocalization was done utilizing the InsituPro VSi pipetting robot adopting the protocol as described previously (Sauer et al., 2006). The anti-PIN2 rabbit antibody (kindly provided by Prof. C. Luschnig) (Paciorek et al., 2005) was used at 1:800 dilution. For the secondary antibody, we used Cy3 anti-rabbit (Sigma-Aldrich) in a 1:600 dilution. For immunolocalization seedlings were grown for four days vertically in Petri dishes on 0.8% agar 0.5 Murashige and Skoog

(MS; www.sigmaaldrich.com) medium containing 1% sucrose (pH 5.7) at 21°C, and under a long-day photoperiod unless otherwise indicated. Prior immunolocalization seedlings were fixed with 4% paraformaldehyde as published previously (Sauer et al., 2006).

**Flavonol content analysis**

The analysis of the flavonol accumulation profile was done as described (Kuhn et al., 2011). Seedlings were grown in a vertical orientation for 6 d as described. One hundred intact seedlings were cut in the hypocotyl region, and roots and shoots were pooled separately, frozen in liquid nitrogen, and lyophilized to determine the dry weight. The dried material was incubated in 500 µL of 80 % methanol overnight at 4° C and subsequently macerated with a pestle, followed by vigorous vortexing. After pelleting the cell debris by centrifugation, the supernatant was transferred to a fresh tube and evaporated in a Speed-Vac centrifuge, with the temperature being limited to a maximum of 42° C. After evaporation, the pellet was resuspended in 100 µL of fresh 80 % methanol and used for analysis. HPLC-ESI-MS and MS/MS experiments were performed on an Acquity UPLC (Waters) connected to a Bruker maXis high-resolution quadrupole time-of-flight mass spectrometer (Bruker Daltonics). An Acquity BEH C18 HPLC column (1.7 µm, 2.1x100 mm fitted with a 2x2 mm guard column) was used with a gradient of solvent A ($H_2O$, 0.1 % (v/v) HCOOH) and solvent B ($CH_3CN$, 0.1 % (v/v) HCOOH), at 0.45 mL flow rate and with a linear gradient from 5 to 95 % B within 30 min.

The mass spectrometer was operated in the negative electrospray ionization mode. MS acquisitions were performed in the full scan mode in the mass range from m/z 50 to 2'000 at 25'000 resolution (full width at half maximum) and 2 scans per second. The MS instrument was optimized for maximum signal intensities of quercitrine at *m/z* 447. Masses were calibrated with a 2 mM solution of sodium formate between *m/z* 180 and 1472 prior to analysis. The lock mass signal of hexakis (1H, 1H, 2H-perfluoroethoxy) phosphazine at *m/z* 556.00195 was further used as lock mass during the HPLC run. Flavonols were identified by the molecular mass determined by MS and by interpretation of fragments obtained by MS/MS, by comparison of both fragmentation patterns and retention times with previous analyses (Ringli *et al.,*

2008), and the absence in flavonol-less *rol1-2 fls1-3* mutants. The area under each flavonol peak was used for relative quantification since it would be difficult to purify each of the flavonol glycosides in large quantities to produce standard curves. The sum of all peak areas represents the relative total amount of flavonols, which was divided by the amount of plant material used for extraction. These values were compared between different plant lines.

### Auxin transport experiments and quantification of derivatives

Arabidopsis mesophyll protoplasts were prepared as described (Geisler et al., 2005) from rosette leaves of plants grown on soil under 100 µM $m^{-2}$ $sec^{-1}$ white light, 8 h light, 16 h dark cycle at 22° C. Intact protoplasts were isolated as described (Geisler et al., 2005) and loaded by incubation with 4-$^{3}$H-1-naphthalene acetic acid (25 Ci $mmol^{-1}$; American Radiolabeled Chemicals) on ice. External radioactivity was removed by separating protoplasts using a 50–30–5 % percoll gradient. Samples were incubated at 25° C and efflux halted by silicon oil centrifugation. Effluxed radioactivity was determined by scintillation counting aqueous phases. Efflux experiments were performed with four to five independent protoplast preparations with four replicas for each time point.

### Gene identifiers of genes used in this study

FLS1 AT5G08640; RCN1 AT1G25490; ROL1 At1G78570

## 4.4 Results

### *rcn1* is a suppressor of the flavonol-induced growth defect of *rol1-2* mutant seedlings

Wild-type Arabidopsis seedlings (ecotype Columbia) develop epinastic cotyledons with puzzle-shaped epidermal pavement cells. By contrast, the *rol1-2* mutant is characterized by hyponastic cotyledons and pavement cells that have lost the typical jigsaw puzzle-like structure (Figure 1). In addition, roots and root hairs of the *rol1-2* mutant are shorter than those of the wild type. To identify suppressor mutants of

*rol1-2*, seeds of this line were mutagenized with ethyl methane sulfonate (EMS) and propagated to the M2 generation (Kuhn et al., 2011). Screening of the M2 seedlings for a suppressed *rol1-2* phenotype resulted in the identification of a number of seedlings with epinastic cotyledons developing a wild type-like pavement cell structure. Some of these revealed a strongly reduced flavonol content and were described earlier (Kuhn et al., 2011), whereas others did not show such a reduction.

Genetic mapping of a recessive, flavonol-accumulating suppressor (for details, see Experimental procedures) allowed narrowing down the mutation to a region on chromosome 1. Since the *rol1-2* mutation is linked to an increase in auxin concentration and a modification of auxin transport, the genetic interval was searched for potential genes known to influence auxin distribution or function, revealing *ROOTS CURL IN NPA 1* (*RCN1*), encoding the regulatory subunit PP2AA1 of the PP2A phosphatase. Sequencing of *RCN1* in the suppressor mutant indeed revealed a point mutation in the coding region introducing a non-sense mutation (Figure 2A). This mutation is subsequently referred to as *rcn1-3*, the first two *rcn1* alleles having been previously identified in the ecotype Wassilewska (Garbers et al., 1996; Larsen and Cancel, 2003). The stop codon in *rcn1-3,* introduced in the codon of Trp471, is at the beginning of the 13$^{th}$ of 15 HEAT (Huntingtin-elongation factor 3-phosphatase subunit A-TOR) repeats. To confirm that the *rcn1-3* mutation is causing suppression of *rol1-2*, the *rol1-2 rcn1-3* double mutant was complemented with a wild-type copy of *RCN1* and with an *RCN1-GFP* construct under the *RCN1* promoter. Several independent transgenic lines were produced that all showed a *rol1-2* mutant phenotype, confirming that *rcn1-3* causes suppression of *rol1-2* (Figure 1G, H).

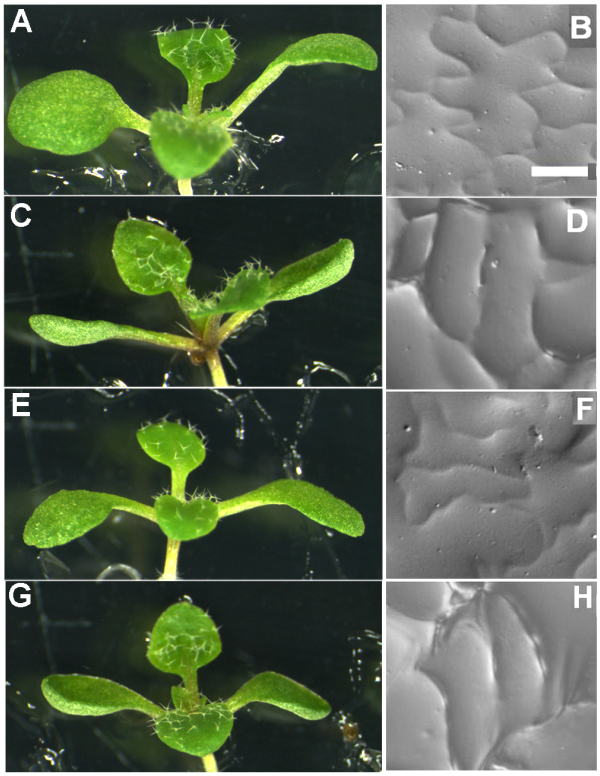



**Figure 1.** *rcn1-3* is a suppressor of the *rol1-2* phenotype. Arabidopsis wild-type plants show an epinastic bending of cotyledons and jigsaw puzzle-like structure of adaxial epidermal pavement cells **(A and B).** The *rol1-2* mutant develops hyponastic cotyledons and distorted, brick-shaped epidermal pavement cells **(C and D).** The *rol1-2 rcn1-3* line shows suppression of the hyponastic growth of cotyledons and partial suppression of the pavement cell shape phenotype **(E and F).** The introduction of a *RCN1:RCN1* genomic clone in a *rol1-2 rcn1-3* line results in the *rol1-2* phenotype **(G and H).** Bar = 1mm **(A, C, E, G),** 40 µm **(B, D, F, H).**

To characterize the *rcn1-3* allele further, an *rcn1-3* single mutant was produced by backcrossing with wild-type Columbia. Similar to the originally identified *rcn1* allele (Garbers et al., 1996), *rcn1-3* seedlings develop shorter roots than wild-type Columbia (Figure 2B), which explains why *rcn1-3* does not suppress the short root-phenotype of *rol1-2* but rather causes a more severe reduction in root length in the *rol1-2 rcn1-3* double mutant, indicative of an additive effect of the two mutations (Figure 2B). By contrast, *rcn1-3* develops a significant portion of wild type-like root hairs which also develop in the *rol1-2 rcn1-3* double mutant. Hence, a fraction of the *rol1-2* root phenotype is suppressed by *rcn1-3* (Figure 2C). The reduced gravitropic response of *rcn1* reported earlier (Rashotte et al., 2001) is also found for *rcn1-3* compared with Columbia and for the *rol1-2 rcn1-3* double mutant compared to *rol1-2* (Figure 2D).

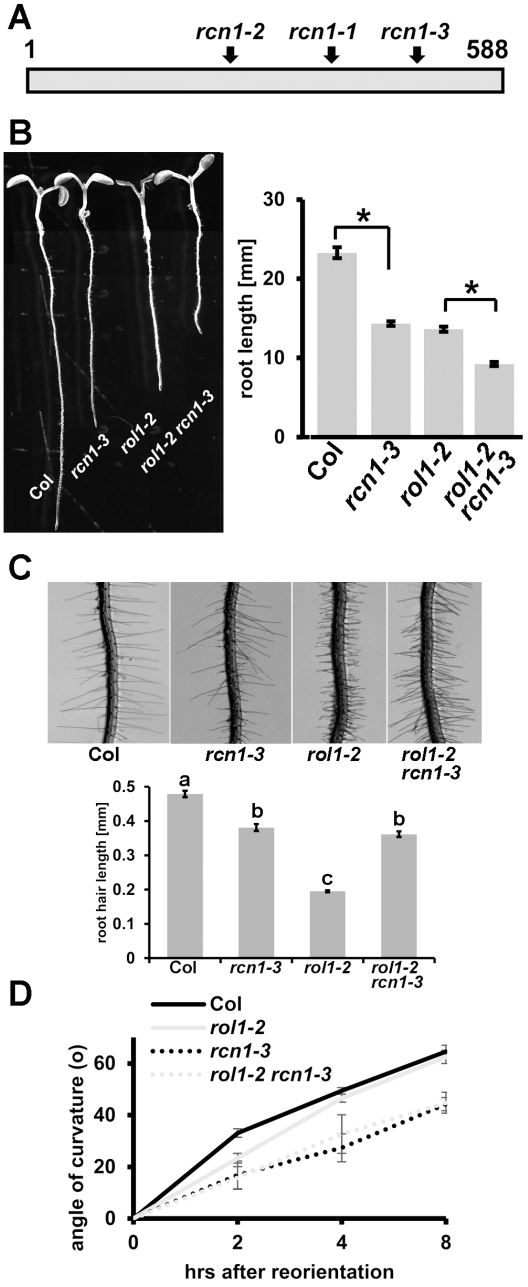



**Figure 2.** Molecular and phenotypic characteristics of *rcn1-3*. **(A)** Schematic representation of the RCN1 protein. *rcn1-1* and *rcn1-2* are a T-DNA insertion and a 23 bp deletion, respectively, in the Arabidopsis Ws-2 background. *rcn1-3* is an EMS-induced nonsense mutation in the Col background. The mutations are at positions corresponding to K352 (*rcn1-1*), Q251 (*rcn1-2*), and W471 (*rcn1-3*). Numbers relate to amino acid positions in the protein. (**B)** The *rcn1-3* mutation causes a reduction in root length as a single mutant and in the *rol1-2* mutant background. Seven days-old seedlings (left) and the statistical analysis (right) are presented. Error bars are shown; asterisks indicate significant differences (n>20; T-test; P<0.001). **(C)** The short root hair-phenotype of the *rol1-2* mutant is alleviated by *rcn1-3*. Root hairs of seven days-old seedlings (top) and the statistical analysis (bottom) are presented. Error bars are shown, different letters on top of the columns indicate significant differences (n=60; T-test; P<0.001). **(D)** Gravitropic response after reorientation of agar plates by 90$^{o}$ was measured at three time points. Error bars are shown, *rcn1-3* containing lines are significantly less gravitropic (n>23; T-test; P<0.001 after 8hrs).

**Phosphatase inhibitors allow for chemical complementation of *rol1-2***

The analyses performed so far suggest that the impaired phosphatase activity due to *rcn1-3* suppresses the *rol1-2* phenotype. To corroborate this hypothesis, phosphatase activity in the *rol1-2* mutant was inhibited by pharmaceutical means. Seedlings of the *rol1-2* mutant were germinated and grown in the presence of the phosphatase inhibitor cantharidin, a terpenoid produced by many beetle species that primarily inhibits PP2A activity (Li and Casida, 1992; Pereira et al., 2011) and has been shown to mimic the *rcn1* mutant phenotype (Deruere et al., 1999). The application of cantharidin led to suppression of the *rol1-2* cell shape phenotype in a dosage dependent manner (Figure 3). *rol1-2* plants grown on media containing 5 µM cantharidin developed a partial suppression resulting in reduced hyponasty of cotyledons, and a cell shape formation comparable to that of the *rol1-2 rcn1-3* line (Figure 1E, F; Figure 3C, D). Increasing the cantharidin concentration to 10 µM fully suppressed the cell shape defect and the hyponastic cotyledons, resulting in wild type-like epidermal pavement cells and epinastic cotyledons (Figure 3E, F). These findings underpin the genetic data that suggest that reducing phosphatase activity suppresses the *rol1-2* mutant shoot phenotype.

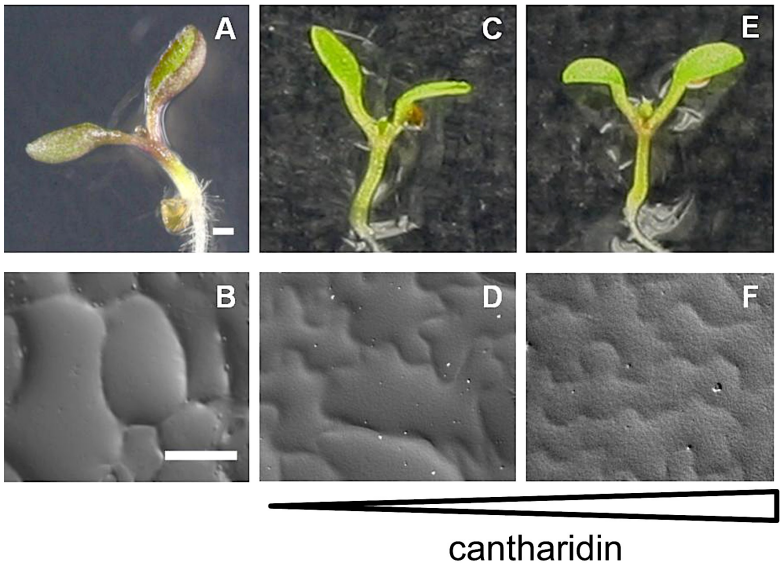



**Figure 3.** Cantharidin treatment of *rol1-2* plants leads to dosage-dependent suppression of the *rol1-2* phenotype. **(A and B)** *rol1-2* mutants show hyponastic cotyledons and defects in pavement cell shape formation. Application of 5 µM Cantharidin on *rol1-2* mutants leads to reduced hyponasty of cotyledons and a partial suppression of the cell shape phenotype **(C and D).** Increasing cantharidin concentration to 10 µM fully suppresses the *rol1-2* phenotype leading to wild type-like bending of cotyledons **(E)** and wild type-like shape of pavement cells **(F).** Bar = 1 mm (**A, C, E),** 40 µm **(B, D, F).**

**Flavonol content is not affected by the *rcn1-3* mutation**

As the *rol1-2* shoot phenotype is strictly dependent on flavonol accumulation, it was important to investigate whether suppression of *rol1-2* by *rcn1-3* was caused by a reduced accumulation of flavonols. To this end, flavonol content of wild-type, *rol1-2*, *rol1-2 rcn1-3* was analyzed by HPLC-MS and signals corresponding to flavonols were identified based on the molecular mass, the comparison with elution profiles done earlier (Ringli et al., 2008; Santelia et al., 2008), and the strong reduction in the flavonol-deficient *rol1-2 fls1-3* double mutant (Kuhn et al., 2011). The area under each flavonol peak was used for relative quantification and the sum of all peak areas represents the relative total amount of flavonols. This revealed that the shoot flavonol content is very similar between the *rol1-2* and the *rol1-2 rcn1-3* mutants and only slightly lower than in the wild type (Figure 4). As expected, the *rol1-2 fls1-3* double mutant contained only very low amounts of flavonols. Hence, *rcn1-3* suppresses the *rol1-2* shoot phenotype by a mechanism that does not involve changes in flavonol accumulation.

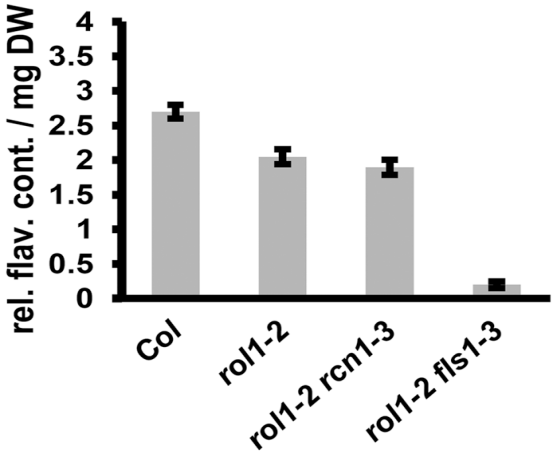



**Figure 4.** Relative flavonol contents in seedlings. *rol1-2 rcn1-3* seedlings show *rol1-2* like flavonol levels in shoots, that correspond to 70% of the wild-type (WT) levels. As a comparison, *rol1-2 fls1-3* mutants are almost devoid of flavonols. Area under the flavonols peaks per mg dry weight was used to quantify flavonol content.

**Auxin transport and PIN2 localization is altered in *rol1-2* and reverted by *rcn1-3***

Previous analyses revealed a modified auxin transport activity in the *rol1-2* mutant, which was induced by the altered flavonol accumulation (Kuhn et al., 2011). This led to the hypothesis that *rcn1-3* suppresses the *rol1-2* shoot phenotype by alleviating the flavonol-induced changes in auxin fluxes without affecting flavonols *per se*. To test this hypothesis, auxin efflux was measured in wild-type, *rol1-2*, and *rol1-2 rcn1-3* employing a protoplast-based auxin transport system (Geisler et al., 2005). The *rol1-2* mutant showed enhanced auxin export compared to the wild type, confirming previous results (Kuhn et al., 2011). *rol1-2 rcn1-3* protoplasts, however, showed a reduction in auxin transport below wild-type levels, thus reverting the *rol1-2* induced effect on auxin transport (Figure 5A). Thus, the *rcn1-3* mutation not only suppresses the *rol1-2* shoot phenotype but also reverts the effect of the *rol1-2* mutation on auxin transport.

By the use of mutants affected in the PP2A regulatory subunits PP2AA1 (RCN1) and either PP2AA2 or PP2AA3, PP2A was shown to influence PIN protein localization via antagonizing the activity of the PID kinase (Michniewicz et al., 2007). Both PP2A and PID modify PIN localization, presumably via the phosphorylation status of these auxin transport proteins. In a first step, it was tested whether localization of PIN proteins is modified in the *rol1-2* mutant. PIN proteins were detected by immunolocalization in root tips, the optimal tissue for this type of analysis. In the majority of young wild-type cortex cells, PIN2 shows basal (lower, towards the tip) localization as previously reported (Kleine-Vehn et al., 2008), resulting in a ratio of apical:basal localization of PIN2 of <1 (Figure 5B, E). By contrast, cortex cells of the *rol1-2* mutant showed mainly apically localized PIN2 (and a ratio of apical:basal localization of PIN2 of >1). The localization of other PIN proteins was not visibly affected. In *rol1-2 rcn1-3* seedlings, however, apicalization of PIN2 is reverted back and a majority of cortical cells revealed to have PIN2 protein on the basal side comparable to the wild type (Figure 5C-E). Hence, the *rcn1-3* mutation reverts the altered PIN2 localization in the *rol1-2* mutant. The *rcn1-3* single mutant showed a wild type-like PIN2 localization (Figure 5E) as previously observed by others (Rahman et al., 2010). To test whether the apicalization of PIN2 in *rol1-2* is induced by flavonols, PIN2 was localized in the flavonol-less *rol1-2 fls1-3* double mutant

(Kuhn et al., 2011). In this line, similar to *rol1-2 rcn1-3*, PIN2 showed wild type-like, predominant basal localization (Figure 5E). This supports the hypothesis that apicalization of PIN2 in *rol1-2* is induced by flavonols and that *rcn1-3* counteracts the flavonol-induced alteration in PIN2 localization.

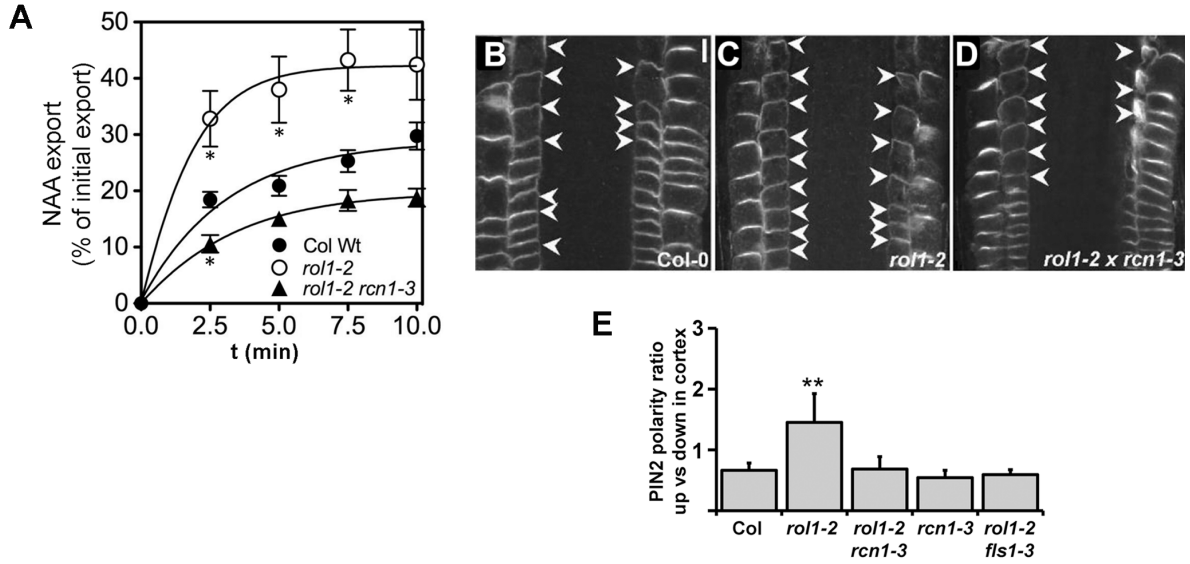



**Figure 5.** Altered auxin transport and PIN2 localization is altered by *rol1-2* and *rcn1-3*. **(A)** Auxin transport activity was measured by quantifying export of NAA from mesophyll protoplasts. Compared to the wild type (Col), *rol1-2* mutant protoplasts show an increased export activity, which is suppressed by in the *rol1-2 rcn1-3* double mutant. N=4, asterisks indicate significantly different from wild type (t-test, $p<0.05$) **(B-D)** Confocal images of Arabidopsis root tips after PIN2 immunolocalization reveal greater number of cortex cells showing apicalization of this auxin efflux carrier in the *rol1-2* mutant allele **(C)** in comparison to the control **(B)** and the rescued PIN2 localization in the *rol1-2 rcn1-3* double mutant **(D)** resembling the control line. Arrowheads indicate apically localized PIN2 in cortex cells. **(E)** The graph represents quantification of PIN2 polarity in cortex cells expressed as a ratio of cells showing apical vs basal localization of this auxin efflux carrier in the different genetic backgrounds **(E);** error bars represent SE for 5 biological repeats (number of seedlings imaged per each replicate. n > 4; double asterisk represents statistically significant difference (P-value < 0.01).

## Flavonols antagonize PID activity *in vivo*

The results obtained so far allow bringing up a model in which flavonols in *rol1-2* modify the phosphorylation status of PIN2 by altering kinase activity, while inhibiting PP2A by the *rcn1-3* mutation brings back this antagonistic kinase/phosphatase activity to a balanced state. This hypothesis is supported by the finding of an inhibitory effect of flavonols on PINOID activity *in vitro* (Henrichs et al., 2012). Here, we aimed at testing the effect of flavonols on PID activity *in vivo* by measuring PID-

induced agravitropism (Benjamins et al., 2001). To this end, transgenic lines were produced containing the genomic coding region of *PID* under the control of the *FLS1* promoter. In Arabidopsis, *FLS1* codes for the main FLAVONOL SYNTHASE committed to the final step in flavonol biosynthesis. Hence, these plants express the transgene-encoded *PID* only in tissue competent to accumulate flavonols. Independent transgenic *FLS1:PID* plants with T-DNA insertion at only one genetic locus were crossed with flavonol-less *fls1-3* mutants to have the very same transgenic events in the two backgrounds. The effect of *FLS1:PID* on plant development was assessed by analyzing gravitropism using the vertical growth index. This method relates absolute root length to progression of root growth along the Y-axis of plants grown in a vertical orientation (Grabov et al., 2005). The value is converted to an angle, which is an accurate measurement of gravitropism (for graphical representation of the method and representative picture of seedlings used for quantification, see Figure S1). This method is particularly useful for plants with rather subtle variations of the gravitropic response. The transgene-conferred PID activity did not have a significant effect in the wild-type background, since transgenic and non-transgenic Columbia showed a gravitropic response that was not significantly different. In the flavonol-less *fls1-3* genetic background, however, *FLS1:PID* induced a significantly reduced gravitropism compared to non-transgenic *fls1-3* seedlings (Figure 7). A change in gravitropism was also observed when comparing non-transgenic Columbia and *fls1-3* mutants, even though the effect was more subtle than in the transgenic lines. These data show that *FLS1*:*PID* has an effect on gravitropism mainly in the absence of flavonols, supporting the hypothesis that the flavonols negatively influence PID activity *in vivo*.

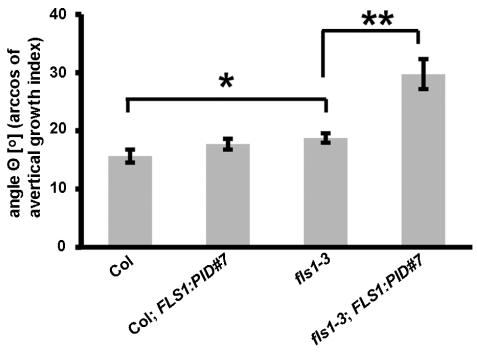



**Figure 6.** Agravitropism induced by *PID* expressed under the *FLS1* promoter. Seedlings were grown for six days in a vertical orientation and the angle Θ, corresponding to the arccos of the vertical growth index (ΔY/L, see Figure S1), was determined for the different lines. The *FLS1:PID* construct

induces agravitropism in the absence of flavonols, indicative of a negative effect of flavonols on PID. *fls1-3*: flavonol-less *flavonol synthase 1* mutant. Error bars are shown, single and double asterisks indicate significant differences of P=0.06 and P<0.001, respectively (n=22).

## 4.5 Discussion

The Arabidopsis *rol1-2* mutant shows growth defects at the seedling stage that are induced by flavonols. These secondary compounds are known to modulate plant growth, the modification of auxin transport being one of the effects of flavonol action (Brown *et al.,* 2001; Buer and Muday, 2004; Jacobs and Rubery, 1988; Kuhn *et al.,* 2011; Peer *et al.,* 2004). The defects in *rol1-2* shoot development are completely suppressed in the absence of flavonols. The short root and short root hair phenotype of *rol1-2*, which is most likely induced by alterations in the cell wall component pectin (Diet et al., 2006), is only slightly alleviated (Kuhn et al., 2011; Ringli et al., 2008). This suggests that the effect of flavonols on root development is camouflaged by the defect in pectin composition, which strongly reduced root growth.

### Flavonols influence the PID/PP2A kinase/phosphatase equilibrium

The *rcn1-3* mutation suppresses the *rol1-2* phenotype without changing flavonol accumulation. The effect of *rcn1-3* on auxin transport and on polar localization of PIN2 in *rol1-2* is comparable to the *fls1-3* mutation that blocks flavonol accumulation (Kuhn et al., 2011), indicating that *rcn1-3* interferes with the effect of flavonols in *rol1-2*. *RCN1* encodes PP2AA-1, one of three regulatory A subunits of the phosphatase PP2A, and mutating *rcn1* reduces phosphatase activity (Deruere et al., 1999). RCN1 might be partially substituted in the *rcn1-3* mutant by one of the two homologous proteins PP2AA-2 and -3 (Michniewicz et al., 2007), which can explain the more complete suppression of the *rol1-2* shoot phenotype by the phosphatase inhibitor cantharidin than by *rcn1-3*. The PP2A phosphatase has been shown to be an antagonist of the PINOID kinase, which also has been identified as a regulator of auxin transport (Benjamins *et al.,* 2001; Christensen *et al.,* 2000). This phosphatase/kinase pair influences the polar localization of PIN auxin transport proteins and auxin transport activity in an antagonistic manner (Huang *et al.,* 2010; Michniewicz *et al.,* 2007; Muday *et al.,* 2006; Rashotte *et al.,* 2001; Sukumar *et al.,* 2009). The *in vitro* kinase activity of PID has recently been shown to be negatively regulated by flavonols (Henrichs et al., 2012). A negative effect of flavonols on PID is

also found *in vivo*, since the *PID* gene under the control of the *FLS1* promoter increases agravitropy particularly in the *fls1-3* mutant background, i.e. in the absence of flavonols. Together, these experiments suggest that flavonols of *rol1-2* negatively regulate PID kinase activity. The loss of kinase activity is compensated for by reducing PP2A phosphatase activity by *rcn1-3* or the phosphatase inhibitor cantharidin, bringing back into balance the (de-) phosphorylation status of targets of PINOID and PP2A activities. This hypothesis is supported by the opposite changes in PIN2 polar localization observed in the *rol1-2* and *rol1-2 rcn1-3* mutants.

### The flavonols of *rol1-2* change the effect of PP2A on PIN2 localization

Previous studies have shown that reducing PP2A activity induces a basal-to-apical shift of PIN2 (Friml et al., 2004; Michniewicz et al., 2007). In the *rol1-2* mutant background, however, *rcn1-3* induces an apical-to-basal shift of PIN2. Thus, the mechanism by which polarization of PIN2 is changed in *rol1-2* and *rol1-2 rcn1-3* is not easily explained with the current understanding of the regulation of PIN polarity. It appears that flavonols in *rol1-2* not only modify PIN2 localization, but also influence the effect of PP2A on PIN2 localization. Hence, flavonols seem to have different entry points at which they modulate the network controlling auxin transport. The increased apical localization of PIN2 in the *rol1-2* mutant would fit with an increased PID activity, since plants containing *35S:PID* show apicalization of PIN2 (Benjamins et al., 2001; Friml et al., 2004). However, flavonols have been shown to inhibit PID activity ((Henrichs et al., 2012); this work). Furthermore, it is unlikely that an increased kinase activity of PID in *rol1-2* would be compensated for by a reduction in PP2A phosphatase activity since both effects would increase phosphorylation levels of substrate proteins.

### Flavonols have effects on multiple targets resulting in changes in auxin transport

The flavonols of *rol1-2* seem not to solely reduce PID activity but are likely to have multiple effects. At the other hand, a change in localization is only detectable for

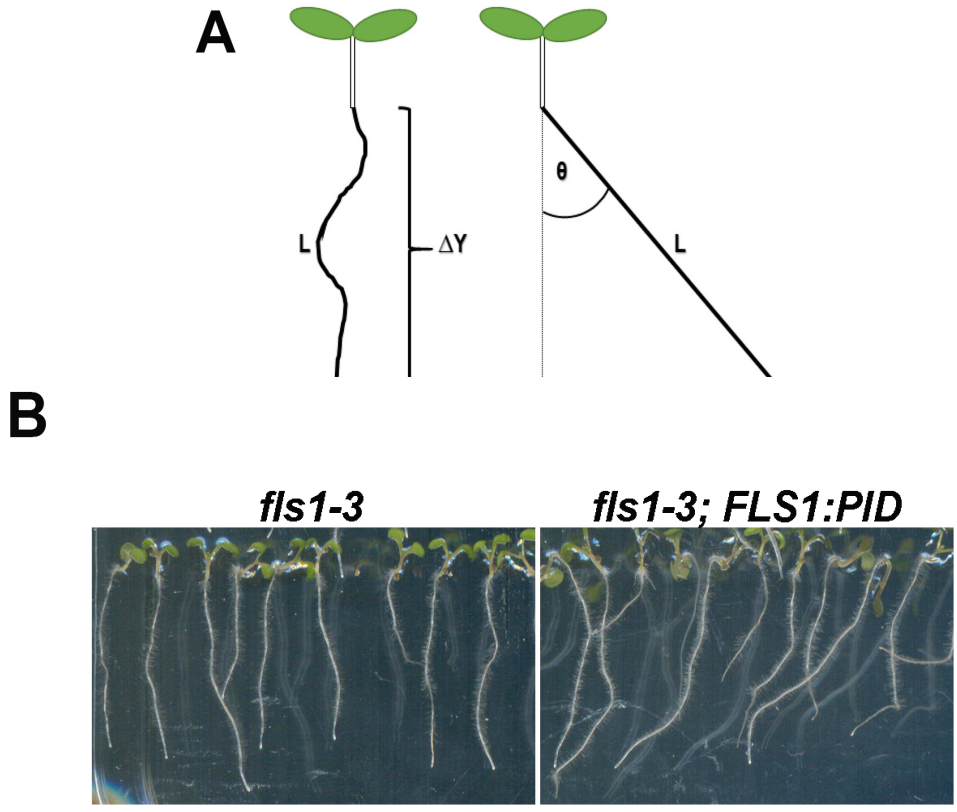
PIN2, suggesting that only a fraction of the machinery determining PIN protein localization is affected.

For example, they might influence other kinases in addition to PID. The PID-related WAG kinases are also implicated in auxin transport (Santner and Watson, 2006) and a family of D6 protein kinases is important for phosphorylation PIN proteins resulting in activation without altering localization of the proteins (Barbosa et al., 2014; Zourelidou et al., 2014). Hence, there are several ways by which flavonols might influence auxin transport. A more detailed analysis will be necessary to investigate their effect on the activity of the individual PIN proteins, and possibly other auxin transporters, such as ABCBs that are also regulated by AGC kinases (Henrichs et al., 2012). Since several kinases are involved, a number of phosphatases are likely to be important to counteract kinase activity. Indeed, RCN1 functions as a regulatory subunit not only for PP2A but also for PP6. PP6 is a PP2A-like phosphatase with homologous catalytic proteins and subunit proteins including RCN1 that are shared with PP2A (Dai et al., 2012; Moorhead et al., 2007). Interfering with PP6 activity by mutating the two catalytic subunits FyPP1 and FyPP2 increases *PIN* expression and induces basal-to-apical shift of PIN proteins including PIN2 in cortical cells of the root. This also causes a number of developmental alterations including reduced interdigitation of pavement cells but extending to defects that are more severe than those of the *rol1-2* or *rol1-2 rcn1-3* mutants (Dai et al., 2012; Li et al., 2011). In terms of PIN2 localization, the effects resulting from interfering with PP6 activity are similar to loss of PP2A activity. The type one protein phosphatase TOPP4 has recently been shown to influence pavement cell formation and PIN1 localization in Arabidopsis (Guo et al., 2015). Hence, several protein phosphatases influence localization of distinct PIN proteins, affect auxin transport, and alter cell shape formation. *rol1-2* shows shoot defects mainly in cotyledons, whereas the alterations in pavement cell formation upon interfering with PP6 and TOPP4 are observed in rosette leaves but not in cotyledons. Hence, a number of phosphatases and kinases function at different times points in development and in different organs, some of which are affected by the flavonols in *rol1-2*, whereas other might be regulated by other means.

This work reveals that the modified auxin transport induced by the altered flavonol accumulation profile in *rol1-2* can be suppressed by reducing phosphatase PP2A activity. The flavonols in *rol1-2* influence PINOID and possibly other kinases, confirming that the balance between antagonistically acting kinase/phosphatase pair(s) influences auxin transporters. At this point, the changes in PIN polarity induced by the flavonols in *rol1-2* and *rol1-2 rcn1-3* remain to be fully understood. Certainly, flavonols influence auxin transport and PIN protein localization in different ways that are not limited to inhibiting PINOID activity.

## Supplementary Data

Supplementary Figure S1: Visualization of the vertical root growth index to quantify gravitropism.




**Supplementary Figure S1.** Visualization of the vertical root growth index to quantify gravitropism. **(A)** The total length of the seedling root (L) and the progression on the Y axis (ΔY) describe the arccosinus of the angle Θ, which is a mathematical description of gravitropic growth. Figure adapted from Grabov et al. (2006). **(B)** A representative example of seedlings grown in a vertical orientation on MS plates used for quantification of the vertical root growth index.

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# 5. 7-Rhamosylated Flavonols Modulate Homeostasis of the Plant Hormone Auxin and Affect Plant development

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which should be cited to refer to this work.

# 7-Rhamnosylated Flavonols Modulate Homeostasis of the Plant Hormone Auxin and Affect Plant Development*

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**Flavonols are a group of secondary metabolites that affect diverse cellular processes. They are considered putative negative regulators of the transport of the phytohormone auxin, by which they influence auxin distribution and concomitantly take part in the control of plant organ development. Flavonols are accumulating in a large number of glycosidic forms. Whether these have distinct functions and diverse cellular targets is not well understood. The *rol1-2* mutant of *Arabidopsis thaliana* is characterized by a modified flavonol glycosylation profile that is inducing changes in auxin transport and growth defects in shoot tissues. To determine whether specific flavonol glycosides are responsible for these phenotypes, a suppressor screen was performed on the *rol1-2* mutant, resulting in the identification of an allelic series of *UGT89C1*, a gene encoding a flavonol 7-O-rhamnosyltransferase. A detailed analysis revealed that interfering with flavonol rhamnosylation increases the concentration of auxin precursors and auxin metabolites, whereas auxin transport is not affected. This finding provides an additional level of complexity to the possible ways by which flavonols influence auxin distribution and suggests that flavonol glycosides play an important role in regulating plant development.**

Flavonoids are secondary metabolites produced via the phenylpropanoid pathway (1), and they serve different functions such as protection from UV irradiation (2), plant-microbe interaction (3), regulation of levels of reactive oxygen species (4), inhibition of cell cycle, and control of cell growth (5, 6). In addition, they are also thought to influence transcriptional and signaling processes (7–9). A major role of flavonols, a subgroup of flavonoids, appears to be the modification of auxin-related processes due to their property of modulating auxin transport (10–14). In some tissues, auxin is transported from cell to cell in a polarized fashion, operated mainly via auxin transporters of the ABCB, AUX1/LAX, and PIN families (15, 16). Flavonoids bind to and inhibit the auxin transport proteins ABCB1 and ABCB19 (17) and interfere with the interaction of these two proteins with the immunophilin-like protein, FKBP42/TWISTED DWARF1 (18). Recent data show that the pinoid kinase, which influences polar localization of PIN proteins (19) and ABCB transport activity, is a likely target of flavonol action (20). The accumulation of flavonols is altered in the *pin2* mutant, the agravitropic phenotype of which can be partially complemented by exogenous flavonols due to their ability to modify expression of *PIN* genes (21–23). Hence, flavonols and auxin are able to mutually influence each other, confirming a functional interdependence in their biological activity.

Flavonoid biosynthesis is well characterized, and a number of mutants affected in the enzymes committed to the different steps have been identified in *Arabidopsis thaliana* (1) (Fig. 1). These lines frequently show a pale yellow seed coat due to the absence of proanthocyanidins and were thus named *transparent testa* (*tt*) mutants (24). Flavonols are produced from dihydroflavonols via the activity of the flavonol synthase, and thus represent a side branch of the flavonoid biosynthesis pathway. In *Arabidopsis*, the flavonols kaempferol, quercetin, and isorhamnetin are glycosylated by one or several sugars, mainly Glc, Rha, and rarely Ara, at the C3 and C7 position of the flavonol backbone (25, 26) through the function of UDP-dependent glycosyltransferases (UGTs),[4] some of which have been identified (8, 26–29). The biological relevance of flavonol glycosylation remains controversial. *In vitro* experiments show activity of flavonol aglycones, suggesting that these are the compounds active in modulating polar auxin transport (10, 17, 18, 20). However, the identification of mutant phenotypes induced by changes in the flavonol glycosylation profile suggests function for at least some flavonol glycosides (30, 31).

Of the major auxin (indole-3-acetic acid; IAA) produced by plants, only a minor fraction of ~1% exists in this active form. Auxin can be conjugated to amino acids and/or sugars (mainly Glc in *Arabidopsis*) for storage in an inactive form or as an initial step for degradation (32, 33). Indole-3-acetyl-alanine (IAA-Ala) and indole-3-acetyl-leucine (IAA-Leu) are readily hydrolyzable, resulting in active free IAA, whereas indole-3-

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[4] The abbreviations used are: UGT, UDP-dependent glycosyltransferases; K-R-3-R-7, kaempferol-3-*O*-7-*O*-rhamnoside; IAA, indole-3-acetic acid; IAA-Glc, IAA-glucose; IAA-Asp, IAA-aspartate; IAA-Glu, IAA-glutamate; oxIAA, 2-oxindole-3-acetic acid; oxIAA-Glc, oxIAA-glucose; IAN, indole-3-acetonitrile; EMS, ethyl methanesulfonate; ESI, electrospray ionization; 1-NAA, 1-naphthalene acetic acid; FLS, flavonol synthase.

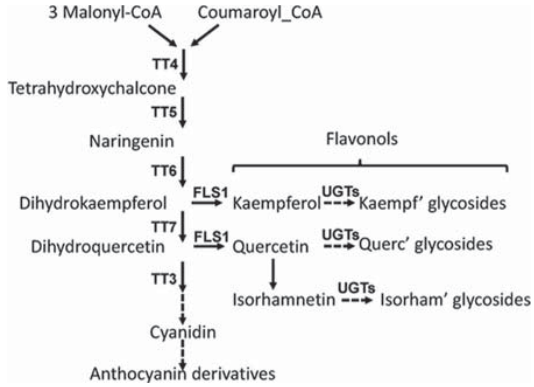


FIGURE 1. **Overview of flavonoid biosynthesis.** The phenylpropanoid pathway leads to the synthesis of flavonoids. These encompass a number of different compounds, not all of which are indicated in this scheme. Enzymes leading to the synthesis of flavonols (kaempferol, quercetin, isorhamnetin) are indicated. TT (transparent testa) proteins were identified based on the pale brown phenotype of seeds of corresponding mutant lines. *FLS1*: flavonol synthase 1, the predominant FLS protein in *Arabidopsis*. *UGTs*: UDP-dependent glycosyltransferases needed for the glycosylation of flavonols.

acetyl-aspartate (IAA-Asp) and indole-3-acetyl-glutamate (IAA-Glu) remain largely stable, suggesting that the dynamics of the reversible (de-)conjugation are decisive in regulating the pool of active auxin. Auxin homeostasis can be affected by mutations in loci involved in auxin (de-)conjugation, and multiple mutations in these loci can induce auxin-related phenotypes ((Ref. 34) and references therein). In *Arabidopsis*, the most abundant degradation products of auxin are produced via oxidation, resulting predominantly in oxIAA (2-oxindole-3-acetic acid) and oxIAA-Glc (2-oxindole-3-acetyl-glucose, glucosyl-ester of oxIAA) (35, 36). This oxidation contributes to the formation of auxin gradients (37) that are crucial for auxin-driven organ development (38 – 41). oxIAA levels are increased in the flavonoid-deficient *tt4* mutant but decreased in the flavonol over-accumulator *tt3* (42). This correlation suggests that the anti-oxidant activity of flavonols (4) influences auxin degradation.

The *rol1-2* (*repressor of lrx1*) mutant is affected in one of three *RHM* genes, *RHM1*, encoding rhamnose synthase, which converts UDP-Glc to UDP-Rha (43). The *rol1-2* mutant was identified in a screen for alterations in cell wall structures and shows modifications in the Rha-rich cell wall component pectin (43). In addition, *rol1-2* also shows changes in the flavonol glycosylation profile, mainly a reduction in the degree of rhamnosylation, whereas the overall quantity of flavonols is not affected (30, 44). This confirms the importance of *RHM1*, which is co-expressed with genes important for flavonol biosynthesis (26) and for flavonol glycosylation. *rol1-2* seedlings are characterized by shorter roots and root hairs than in the wild type. The *rol1-2* seedling shoot develops deformed trichomes and hyponastic cotyledons with brick-shaped pavement cells, whereas the wild type develops regular trichomes and epinastic cotyledons with puzzle-shaped jigsaw-like pavement cells (30, 43). There is no obvious growth defect detectable in *rol1-2* adult plants, presumably due to the functional redundancy among the three *RHM* genes (45).

The short root phenotype of the *rol1-2* mutant is most likely induced by the changes in pectin structures (43). By contrast, the aberrant shoot phenotype of the *rol1-2* mutant is connected to the altered flavonol composition. Mutations affecting steps in flavonoid biosynthesis (Fig. 1) upstream of flavonols such as *tt4* or *tt6*, mutations in the *flavonol synthase* (*FLS1*), or mutations in a positive regulator of flavonol biosynthesis encoded by *MYB111* all suppress *rol1-2*, resulting in wild type-like shoot development, whereas root development is comparable with the *rol1-2* single mutant (30, 44). A mutation in *TT7*, blocking quercetin accumulation, or any step farther downstream in the pathway has no effect on the *rol1-2* shoot phenotype (30, 44). Together, these findings suggest that the *rol1-2* phenotype is induced by the accumulation of flavonol glycosides that interfere with proper plant development and that kaempferols are sufficient to induce this defect. Yin *et al.* (31) have shown that the dwarf growth phenotype of the flavonoid 3-*O*-glucosyltransferase mutant *ugt78d2* correlates with the over-accumulation of 3-*O*-7-*O*-rhamnosylated kaempferol (K-R-3-R-7), and interfering with K-R-3-R-7 biosynthesis suppresses the growth defect of the *ugt78d2* mutant. By contrast, the flavonol species inducing the *rol1-2* phenotype is most likely not K-R-3-R-7, because this compound is present in lower amounts than in the wild type. Thus, it is likely that several flavonol glycosides can have an effect on plant development. Both *rol1-2* and *ugt78d2* show changes in auxin concentration or transport activity. For *rol1-2*, it was shown that auxin transport is reverted to wild-type levels when blocking flavonol accumulation (44), thus indicating that flavonols do interfere with auxin distribution.

Aiming at the identification of flavonol glycosides that induce the *rol1-2* phenotype, EMS-induced suppressor mutants of *rol1-2* were selected for specific changes in flavonol glycosylation. Several alleles of the 7-*O*-rhamnosyltransferase locus *UGT89C1* were identified. The auxin transport activity in *rol1-2* is not changed by a *ugt89c1* mutation, but the levels of auxin conjugates and catabolites are strongly increased in the *ugt89c1* mutant background. This indicates that flavonols affect not only auxin transport but also auxin turnover, and in this way modify auxin homeostasis.

## Experimental Procedures

*Plant Material, Growth Conditions, EMS Mutagenesis, and Mutant Screen*—All lines described in this study are in the Col-0 genetic background. The *rol1-2* allele and *fls1-3* allele used in this study are described elsewhere (43, 44). For all analyses described, the *ugt89c1-3* nonsense allele was used. Seeds were surface-sterilized with 1% sodium hypochlorite, 0.03% Triton X-100, plated on half-strength Murashige and Skoog medium containing 0.6% Phytagel, 2% sucrose, 100 $\mu$g/ml *myo*-inositol, stratified for 4 days at 4 °C, and grown in a vertical orientation in a 16-h light, 8-h dark cycle at 22 °C. For propagation and crossings, 10-day-old plants were transferred to soil, grown in a 16-h light, 8-h dark cycle at 22 °C, and irradiated with white light (Biolux, Osram). The EMS screen was performed as described (44), and 75,000 M2 seedlings were screened for a suppressed *rol1-2* mutant phenotype. All *ugt89c1* alleles were backcrossed at least three times to Col-0 and *rol1-2* prior to analysis. Plant transformation was done as

described (43), and transgenic plants were selected with BASTA (10 $\mu$g/ml).

*DNA Constructs, Plant Transformation, and Molecular Markers*—For the *UGT89C1* complementation construct, the *UGT89C1* genomic clone was PCR-amplified with the primers *F7RT_PC* 5′-GGCGCGCCAGACTACAGTTTGGCTAACCAG-3′ and *F7RT_R3C* 5′-TGAACCGCGTGTGTAATGTATC-3′, encompassing 1.5-kb promoter, 1.3-kb coding sequence, and 0.35-kb terminator sequence. The resulting fragment (*UGT89C1:UGT89C1*) was cloned into pGEM-T Easy (Promega) for sequencing. For the *GFP* fusion construct, a BamHI site was introduced into *UGT89C1:UGT89C1* clone by PCR 3′ of the *ATG* (N-terminal fusion). PCR was performed with the primers *F7RT_R4_NGFP* 5′-GGATCCCATGATTGATGTTTTTTTCTTTC-3′ and *F7RT_F6_NGFP* 5′-GGATCCACAACAACAACAACGAAGAAGC-3′. A previously produced *GFP* construct flanked by BamHI sites (46) was cloned into this BamHI site, resulting in *UGT89C1:GFP-UGT89C1*. This construct was cloned into the binary vector *pBART* (47) by NotI.

The molecular markers for the *rol1-2* and *fls1-3* mutations are described elsewhere (43, 44). For detection of *ugt89c1-3*, a PCR was performed using the primers *F7RT_F4* 5′-TGATGCTTTCTCTATTAAGTCCAT-3′ and *F7RT_R5_CGFP* 5′-GGATCCCAAACACATCTCTGCAACGAG-3′. The PCR product was cut by ApaI (cuts wild type) and run on a 1.5% agarose gel. For detection of *ugt89c1-11*, PCR was performed using the primers *F7RT_F4* 5′-TGATGCTTTCTCTATTAAGTCCAT-3′ and *F7RT_R5_CGFP* 5′-GGATCCCAAACACATCTCTGCAACGAG-3′. PCR was performed at 55 °C annealing temperature, 1.5 mM $MgCl_2$, and 1 min of elongation time, with 35 cycles. The PCR product was cut by BglII (cuts mutant) and run on a 1.5% agarose gel. All other *ugt89c1* alleles were confirmed by sequencing.

*Microscopic Analysis*—Phenotypic screening for and analysis of *ugt89c1 rol1-2* was performed using a binocular microscope. GFP fluorescence was photographed using a Leica DM6000 stereomicroscope. Gel prints of epidermal cells were produced following an established protocol (48) and observed by differential interference contrast microscopy using a Leica DMR microscope.

*Flavonol Content Analysis*—The analysis of the flavonol accumulation profile was done as described (44). Seedlings were grown in a vertical orientation for 6 days as described. One hundred intact seedlings were cut in the hypocotyl region, and roots and shoots were pooled separately, frozen in liquid nitrogen, and lyophilized to determine the dry weight. The dried material was incubated in 500 $\mu$l of 80% methanol overnight at 4 °C and subsequently macerated with a pestle, followed by vigorous vortexing. After pelleting the cell debris by centrifugation, the supernatant was transferred to a fresh tube and evaporated in a SpeedVac centrifuge, with the temperature being limited to a maximum of 42 °C. After evaporation, the pellet was resuspended in 100 $\mu$l of fresh 80% methanol and used for analysis. HPLC-ESI-MS and MS/MS experiments were performed on an ACQUITY UPLC (Waters) connected to a Bruker maXis high-resolution quadrupole time-of-flight mass spectrometer (Bruker Daltonics). An ACQUITY BEH C18 HPLC column (1.7 $\mu$m, 2.1 × 100 mm fitted with a 2 × 2-mm guard column) was used with a gradient of solvent A ($H_2O$, 0.1% (v/v) HCOOH) and solvent B ($CH_3CN$, 0.1% (v/v) HCOOH), at 0.45-ml flow rate and with a linear gradient from 5 to 95% B within 30 min.

The mass spectrometer was operated in the negative electrospray ionization mode. MS acquisitions were performed in the full scan mode in the mass range from *m/z* 50 to 2,000 at 25,000 resolution (full width at half-maximum) and two scans per second. The MS instrument was optimized for maximum signal intensities of quercitrine at *m/z* 447. Masses were calibrated with a 2 mM solution of sodium formate between *m/z* 180 and 1,472 prior to analysis. The lock mass signal of hexakis (1*H*,1*H*,2*H*-perfluoroethoxy)phosphazene at *m/z* 556.00195 was further used as lock mass during the HPLC run. Flavonols were identified by the molecular mass determined by MS and by interpretation of fragments obtained by MS/MS, by comparison of both fragmentation patterns and retention times with previous analyses (30), and by their absence in flavonol-less *rol1-2 fls1-3* mutants (44). Purification of each flavonol glycoside in sufficient quantities for producing standard curves was not possible. Therefore, the content of each individual flavonol was expressed as the peak area of the corresponding high-resolution extracted ion chromatogram. The total flavonol content was calculated by summing up all flavonol peak areas, divided by the weight of plant material used for extraction. Either the total or the individual flavonol contents were compared between different experiments.

*Auxin Transport Experiments and Quantification of Derivatives—Arabidopsis* mesophyll protoplasts were prepared as described (49) from rosette leaves of plants grown on soil under 100 $\mu$M $m^{-2}$ $s^{-1}$ white light, 8-h light, 16-h dark cycle at 22 °C. Intact protoplasts were isolated as described (50) and loaded by incubation with 1-[4-$^3$H]naphthalene acetic acid (25 Ci $mmol^{-1}$; American Radiolabeled Chemicals) on ice. External radioactivity was removed by separating protoplasts using a 50–30-5% Percoll gradient. Samples were incubated at 25 °C, and efflux was halted by silicon oil centrifugation. Effluxed radioactivity was determined by scintillation counting of aqueous phases and is presented as the relative efflux of the initial efflux (efflux prior to incubation), which was set to zero. Efflux experiments were performed with 3–5 independent protoplast preparations with four replicas for each time point. Basipetal (shoot-ward) auxin transport was measured according to Ref. 51. Radioactive auxin ([$^3$H]IAA) was applied to the root tips of 15 seedlings ($n = 4$), and 5–10-mm segments were cut after 18 h and counted. Segments in the wild-type Col were set as 100%, and others were calculated accordingly.

Measuring the concentration of auxin, auxin conjugates, and auxin degradation products was done as follows. The determination of IAA and its metabolites included extraction and purification (according to Ref. 52) followed by quantitation using LC-MS/MS (as described in Ref. 53). Briefly, a sample (about 100 mg of fresh weight) was homogenized in liquid nitrogen. The homogenate was supplied with 500 $\mu$l of cold extraction buffer (methanol/water/formic acid, 15/10/5, v/v/v, −20 °C) and with a mixture of stable isotope-labeled internal standards: [$^{13}C_6$]IAA (Cambridge Isotope Laboratories) and

$[^2H_5\text{-}^{15}N_1]$IAA-Asp, $[^2H_5\text{-}^{15}N_1]$IAA-Glu, and $[^2H_2]$OxIAA (OlChemIm, Olomouc, Czech Republic); 10 pmol each. After incubation for 30 min at −20 °C, the extract was centrifuged at 28,000 × *g* (centrifuge Eppendorf 5430 R, Eppendorf AG, Hamburg, Germany), and pellet was re-extracted once. Pooled supernatants were evaporated in vacuum concentrator (Alpha RVC, Martin Christ, Osterode am Harz, Germany), and then a sample residue was dissolved into 0.1 M formic acid and applied to mixed mode reversed phase-cation exchange SPE column (Oasis-MCX, Waters). Auxin and its metabolites were eluted with methanol. Eluate was evaporated to dryness in a vacuum concentrator and dissolved into 30 μl of 15% acetonitrile. An aliquot was analyzed on an HPLC (Ultimate 3000, Dionex) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems) set in selected reaction monitoring mode. Quantification of hormones was done using the isotope dilution method with multilevel calibration curves ($r^2 > 0.99$). Data processing was carried out with Analyst 1.5 software (Applied Biosystems).

*Accession Numbers*—Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank™/European Molecular Biology Laboratory (EMBL) databases under the following accession numbers: *FLS1*, At5g08640; *ROL1*/*RHM1*, At1g78570; *UGT89C1*, At1g06000.

## Results

*Altered Flavonol Glycosylation Suppresses the rol1-2 Shoot Growth Defect*—The *rol1-2* shoot phenotype is characterized by hyponastic cotyledons, brick-shaped adaxial pavement cells of cotyledons, and deformed trichomes (Fig. 2*A*). Mutations in a glycosyltransferase involved in producing the *rol1-2* phenotype-inducing flavonol(s) would be expected to revert the *rol1-2* phenotype and act as suppressor(s) of the *rol1-2* phenotype. Thus, to identify possible *rol1-2* phenotype-inducing flavonols, *rol1-2* seeds were mutagenized with ethyl methanesulfonate. After mutagenesis, the seeds were propagated, and seedlings of the M2 generation were screened for a wild-type shoot phenotype that is characterized by epinastic cotyledons with puzzle-shaped pavement cells and upright, branched trichomes (Fig. 2*A*). The identified lines were propagated and confirmed in the M3 generation. Positive lines were backcrossed with *rol1-2*, and only those with an F2 segregation pattern indicating recessive suppressor mutations were further analyzed. Several lines were identified that suppressed the *rol1-2* phenotype, yet showed accumulation of flavonols to levels comparable with the wild type. Among these lines, only one group exhibited the lack of one specific type of flavonols, namely those rhamnosylated at the C7 position. Based on this finding, the previously identified flavonol 7-rhamnosyltransferase gene *UGT89C1* (29) was identified as a potential candidate suppressor gene, and sequencing of the *UGT89C1* locus At1g06000 indeed led to the identification of a mutation in this gene in all these mutants. In total, nine different *ugt89c1*-alleles were identified. Because two T-DNA insertion alleles of *ugt89c1* were already described, the newly identified EMS alleles were numbered *ugt89c1-3* to *ugt89c1-11*. In total, six missense and three nonsense mutations were identified (Table 1). The *ugt89c1-3* and *ugt89c1-6* alleles are affected in the same codon, but the resulting nonsense mutations originate from two different mutation events. Hence, the *ugt89c1-3* and *ugt89c1-6* lines represent two independent alleles with the same effect at the protein level. All mutant *ugt89c1* alleles identified in this study lead to a comparable suppression of the *rol1-2* shoot phenotype, resulting in seedlings with epinastic cotyledons, jigsaw puzzle-shaped pavement cells, and wild type-like trichomes (Fig. 2*A*).

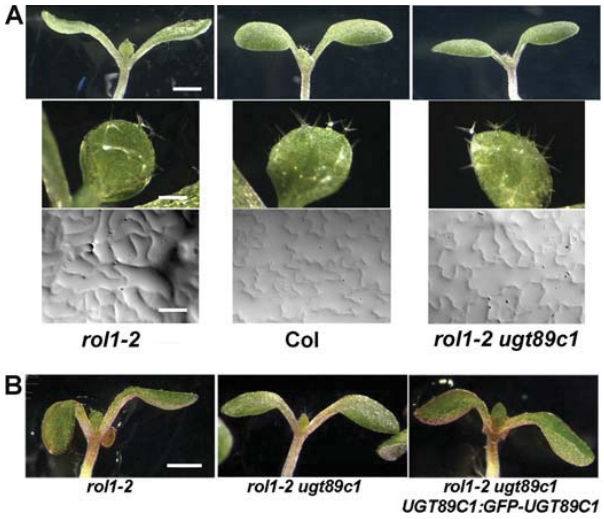


FIGURE 2. **The *rol1-2* mutant shoot cell growth phenotype is suppressed by mutations in *UGT89C1*.** *A*, the *rol1-2* mutant shows hyponastic bending of cotyledons, distorted trichomes, and brick-shaped adaxial pavement cells, whereas the wild type (*Col*) shows epinastic bending of cotyledons, normal shaped trichomes, and a jigsaw puzzle-like structure of adaxial pavement cells. All *ugt89c1* alleles identified in this study suppress the *rol1-2* shoot phenotype, *i.e. rol1-2 ugt89c1* double mutants develop comparable with the wild type. Pictures of the *rol1-2 ugt89c1-3* line are shown that are representative for all other *ugt89c1* lines. *B*, the *UGT89C1:GFP-UGT89C1* construct complements the *ugt89c1* mutation, and hence encodes a functional UGT89C1 protein, resulting in a *rol1-2* mutant phenotype. *Bars* = 1 mm for shoot (*A*, *upper panels*, and *B*); 0.2 mm for trichomes (*A*, *middle panels*); and 40 μm for pavement cells (*A*, *lower panels*).

*ugt89c1 Mutants Specifically Lack 7-Rhamnosylated Flavonols*—A detailed analysis of the flavonol content of *rol1-2 ugt89c1* mutants was performed by HPLC-MS. Individual flavonols were identified by comparing the data with previous studies where flavonols were identified by HPLC-MS/MS, correlation with reference compounds, and the absence in a flavonol-deficient *flavonol synthase1* (*fls1*) mutant background (30, 44). For these and all subsequent analyses, the *ugt89c1-3* nonsense allele was used. The quantification of flavonol accumulation using the sum of areas of all identified flavonol-peaks in HPLC-MS analyses confirmed that the overall flavonol content of the *rol1-2 ugt89c1-3* line is comparable with the wild type or the *rol1-2* mutant (Fig. 3*A*). As a negative control, the *rol1-2 fls1-3* double mutant was included that shows a strongly reduced flavonol content due to a lesion in the *flavonol synthase 1* gene (44). The quantification of individual flavonol peaks confirmed the absence of all identifiable 7-*O*-rhamnosylated flavonols in *rol1-2 ugt89c1* mutants. Other flavonol glycoside derivatives were still present, some at levels higher than in the wild type or *rol1-2* single mutant, as shown for the kaempferol-3-*O*-glucoside (Fig. 3*B*). Flavonols of root and shoot tissue are

**TABLE 1**
**List of *ugt89c1* alleles identified in this screen**

| Allele name | Mutation |
|---|---|
| *ugt89c1-3* | Stop 314[a] |
| *ugt89c1-4* | G123R |
| *ugt89c1-5* | S19F |
| *ugt89c1-6* | Stop 314[a] |
| *ugt89c1-7* | G249V |
| *ugt89c1-8* | Stop335 |
| *ugt89c1-9* | D356N |
| *ugt89c1-10* | T4I |
| *ugt89c1-11* | R253W |

[a] The mutations producing the stop codon are not identical.

affected, confirming the ubiquitous importance of UGT89C1 for flavonol 7-*O*-rhamnosylation. These data indicate a lack of genetic redundancy in *Arabidopsis* with respect to 7-*O*-rhamnosylation of flavonols. Hence, UGT89C1 appears to be the only enzyme that is capable of catalyzing this reaction to a significant extent. The lack of 7-*O*-rhamnosylated flavonols in *rol1-2 ugt89c1-3* suggests an important role of these compounds in the induction of the *rol1-2* shoot phenotype.

*Different Protein Domains Are Affected in the ugt89c1 Alleles*—Analysis of the UGT89C1 protein sequence was chosen as a strategy to elucidate the effect of the different mutations on the secondary and tertiary protein structure. UGT89C1 was compared with the flavonol-specific UGTs *Vv*GT1 from *Vitis vinifera* and *Mt*71G1 of *Medicago truncatula* whose secondary and tertiary structures are known (54, 55). *Vv*GT1 and *Mt*71G1 accept flavonols as donor substrates and therefore were selected for sequence and structural comparison. Previous analysis of four plant UDP-dependent glycosyltransferases revealed a low overall conservation on the protein sequence level, but a high conservation on the level of secondary and tertiary protein structure (56). The alignment (Fig. 4) shows only one short region of 45 amino acids, the PSPG domain that is relatively well conserved and important for binding of the UDP-sugar substrate. All three nonsense alleles and the missense allele *ugt89c1-9*, introducing an D356N substitution, affect residues in this PSPG domain. Mutational analysis has shown that the Asp residue affected in *ugt89c1-9* is essential for function of *Vv*GT1 (55). Of the remaining five alleles, four alter the N1- or C1-loops (*ugt89c1-5*, *-10*, and *ugt89c1-7*, *-11*, respectively) that are involved in forming the sugar donor binding pocket (56). Altogether, protein sequence analysis revealed that a vast majority of mutations that revert the *rol1-2* phenotype are directly related to sugar donor binding on the UGT89C1 enzyme.

*UGT89C1 Expression Profile*—The *rol1-2* cell growth phenotype in cotyledons is characterized by brick-shaped pavement cells on the adaxial side. Previous studies revealed that the flavonol synthase *FLS1* is expressed asymmetrically on the adaxial side (44), providing an explanation for the limitation of the flavonol-related cell growth defect to this side of the cotyledons. To investigate the localization of UGT89C1, a *GFP-UGT89C1* construct under the control of the *UGT89C1* promoter was produced and transformed into *Arabidopsis*. Complementation of the *rol1-2 ugt89c1-3* mutant was used to assess the impact of GFP on UGT89C1 protein activity. In the T2 generation, transgenic plants developed the typical *rol1-2* shoot growth phenotype (Fig. 2*B*), confirming complementation and

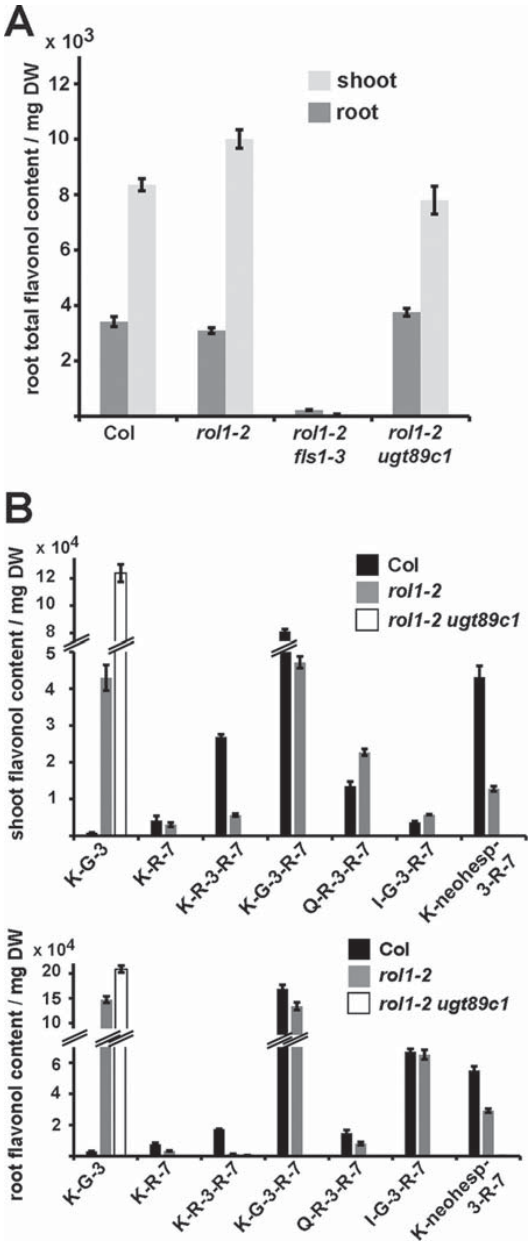


FIGURE 3. **Content of total flavonols and of 7-*O*-rhamnosylated flavonols.** *A*, total flavonol accumulation is comparable in the wild type, *rol1-2*, and *rol1-2 ugt89c1-3*. Values of *rol1-2 fls1-3*, which fail to accumulate flavonols, serve as negative control. *DW*, dry weight. *B*, the *rol1-2 ugt89c1-3* line shows a loss of 7-rhamnosylated flavonols, whereas *e.g.* 3-*O*-glucosylated flavonols are still present and accumulate both in the shoot (*upper graph*) and in the root (*lower graph*). Only a sub-selection of flavonols is shown. For quantification, the area under each peak of the HPLC elution profile was used as an arbitrary unit. *K-G-3*, kaempferol-3-*O*-glucoside; *K-R-7*, kaempferol-7-*O*-rhamnoside; *K-G-3-R-7*, kaempferol-3-*O*-glucoside-7-*O*-rhamnoside; *Q-R-3-R-7*, quercetin-3-*O*-7-*O*-rhamnoside; *I-G-3-R-7*, isorhamnetin-3-*O*-glucoside-7-*O*-rhamnoside; *K-neoh-3-R-7*, kaempferol-3-*O*-neohesperidoside-7-*O*-rhamnoside. *Error bars* indicate mean ± S.E.

thus activity of the GPF-UGT89C1 fusion protein. To assess protein localization, the *UGT89C1:GFP-UGT89C1* construct was transformed into wild-type *Arabidopsis* and GFP fluores-

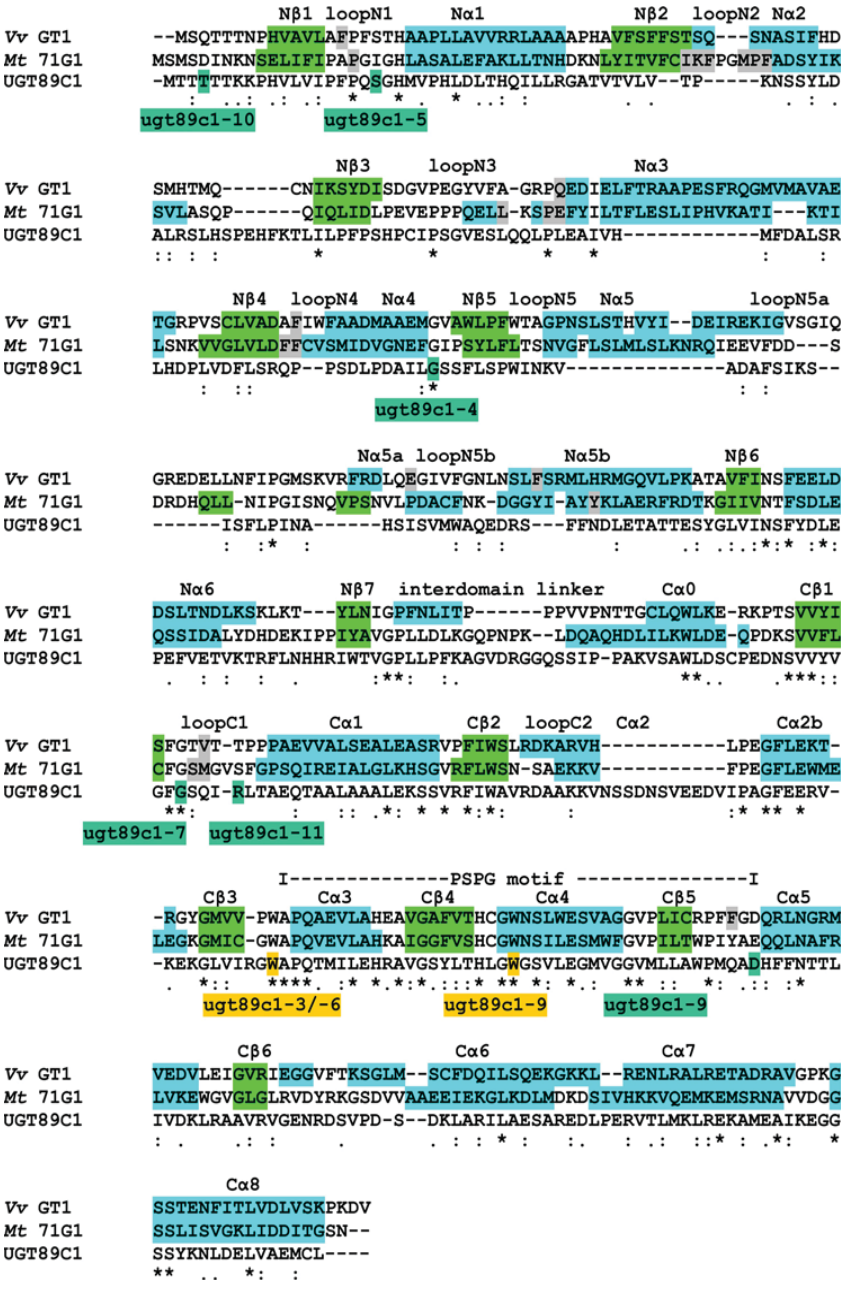


FIGURE 4. **Structural alignment of primary sequences of different UGTs with UGT89C1.** The crystal structure of VvGT1 (*V. vinifera* (*Vv*)) and Mt71G1 (*Medicago truncatula* (*Mt*)) was already solved, and different domains are marked with colors. α-Helices are highlighted in *blue*, and β-strands are in *light green*. The structurally important loops are labeled. Nonsense mutations identified in this screen are marked in *beige*, and missense mutations are in *dark green*. The *dashed line* indicates the PSPG motif important for UDP-sugar donor specificity. Residues reported to be involved in acceptor pocket formation are highlighted in *gray*. *Asterisks* mark identity, *colons* mark high similarities, *single dots* mark lower similarities, and *blank positions* indicate no similarities between the amino acids in the compared sequences. Adopted from Ref. 56.

cence was analyzed in seedlings of the T2 generation of several independent transgenic lines. GFP fluorescence could be detected on the adaxial side of cotyledons and emerging leaves (Fig. 5*A*, C), comparable with the previously reported pattern observed in *FLS1:GFP-FLS1* (Fig. 5*B*) and *RHM1:RHM1-GFP* (*RHM1* is identical to *ROL1*) transgenic lines (44). Trichomes also showed a strong GFP fluorescence (Fig. 5*G*). In roots, GFP fluorescence was predominant in columella cells and in the late elongation/early differentiation zone, correlating with zones of high auxin concentrations. Subcellularly, GFP-UGT89C1 localizes to the cytoplasm and the nucleus (Fig. 5, *D–F*). The expression pattern of *GFP-UGT89C1* is similar to *FLS1* (44) confirming the previous finding that *UGT89C1* is co-expressed with other genes coding for enzymes of the flavonoid biosynthesis pathway (29).

*Auxin Distribution but Not Auxin Transport Is Modified by the ugt89c1 Mutations*—Flavonols have been shown to modify auxin-related processes (13, 14). *rol1-2* mutant seedlings

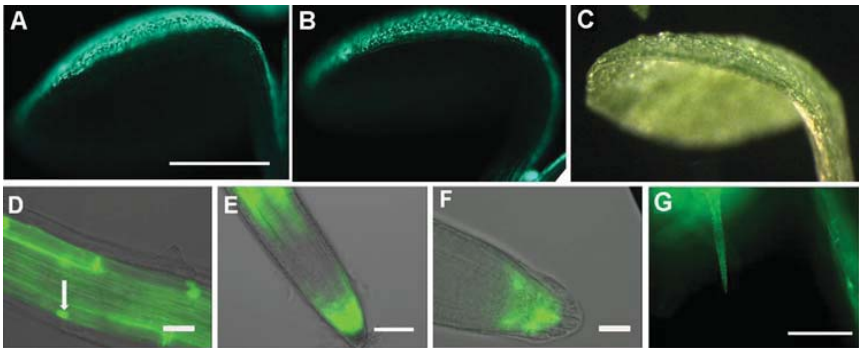


FIGURE 5. **Localization of GFP-UGT89C1.** *A* and *B*, in cotyledon of 6-day-old seedlings, fluorescence induced by *UGT89C1:GFP-UGT89C1* (A) is restricted to the adaxial side, very similar to seedlings containing *FLS1:FLS1-GFP* (B). *C*, bright field image of a cotyledon corresponding to those shown in *A* and *B*. In roots, fluorescence of GFP-UGT89C1 was observed in the cortex and vascular cell layers of the differentiation zone. *D*, on the subcellular level, a fraction of the fusion protein localizes to the nucleus (*arrow*). *E*, in root tips, fluorescence occurred around the meristematic and later elongation zone. *F*, detailed analysis of root tips revealed *GFP-UGT89C1* expression in columella and lateral root cap cells. *G*, GFP-UGT89C1 induced fluorescence in trichomes. *Bars* = 1 mm (*A–C*); 15 $\mu$m (*D* and *F*); 30 $\mu$m (*E*); and 100 $\mu$m (*G*).

indeed show an altered auxin distribution and transport activity, as determined by auxin concentration measurements and transport assays (30, 44). To determine whether *ugt89c1* functions as a suppressor of *rol1-2* by influencing auxin distribution, auxin export was measured in the wild type, *rol1-2*, and *rol1-2 ugt89c1* mutant lines. Due to the strong growth defect of *rol1-2* mutant seedlings, which might interfere with interpretation of auxin transport in a whole-seedling assay, rosette leaf mesophyll protoplasts were used following an established protocol (44). In a first step, flavonols were extracted from wild-type, *rol1-2*, and *rol1-2 ugt89c1* protoplasts to confirm that the mutations have an effect on flavonol accumulation in this cell type. Comparable with flavonols of seedlings, flavonols of *rol1-2* protoplasts revealed a glycosylation profile that is distinct from wild-type protoplasts in that they accumulate fewer rhamnosylated and more glucosylated flavonols (Fig. 6*A*) (30). *rol1-2 ugt89c1* protoplasts did not accumulate 7-*O*-rhamnosylated flavonols. Hence, protoplasts of the different genotypes showed the expected flavonol glycosylation profile, making them suitable to investigate the effect of the two mutations on auxin transport. Auxin export rates were determined in protoplasts using the synthetic auxin, 1-NAA, and revealed that the *rol1-2* mutant shows a modified transport efficiency as compared with the wild type. *rol1-2 ugt89c1-3* double mutant protoplasts, however, show a transport rate that is not different from the one of *rol1-2* (Fig. 6*B*). Hence, mutations in *ugt89c1* suppress the *rol1-2* phenotype by an auxin transport-independent manner. This is particularly interesting because the flavonol-less *rol1-2 fls1* mutant suppresses *rol1-2* by changing auxin transport back to wild-type levels (44). Hence, the mode of action of *fls1* and *ugt89c1* is different. To sustain cellular auxin transport data and to back up measurements performed with synthetic auxin 1-NAA, we measured basipetal (shoot-ward) IAA transport in roots. Comparable with 1-NAA transport in protoplasts, root basipetal transport of IAA was revealed to be changed in *rol1-2* as compared with the wild type. The *rol1-2 ugt89c1* double mutant shows transport activity similar to *rol1-2*, again indicating that *ugt89c1* does not modify the *rol1-2*-induced alteration in auxin transport (Fig. 6*C*). To investigate whether *ugt89c1* changes auxin levels in a different way, concentrations of free auxin and auxin-related metabolites were determined in wild type, *rol1-2*, and *rol1-2 ugt89c1* mutant seedlings. Here, two additional lines were included: the *ugt89c1* single mutant to confirm effects found for *rol1-2 ugt89c1*; and the *rol1-2 fls1-3* double mutant to obtain indications of whether *ugt89c1* and *fls1-3* indeed suppress *rol1-2* by different means. The levels of the prevailing auxin of *Arabidopsis*, IAA, was subject to variation between several biologically independent experiments. As a consequence, the increase in auxin concentration in *rol1-2* as compared with the wild type was not as pronounced as reported previously (30) and did not fulfill the criteria of statistical significance (*t* test, $p < 0.05$). The reason for the observed variation is not clear at this point. Quantification of the auxin precursor indole-3-acetonitrile (IAN) and auxin conjugates and degradation products, on the other hand, gave very consistent results: *ugt89c1* shows increased accumulation of IAN as well as of auxin conjugates and degradation intermediates such as IAA-Glc or oxIAA-Glc (Fig. 7). This effect of *ugt89c1* is observed not only in the context of the *rol1-2* mutation that causes aberrant accumulation of flavonols, but also in the *ugt89c1* single mutant as compared with the wild type, suggesting that the accumulation of auxin precursor and derivatives is genuinely affected by flavonol glycosides.

## Discussion

Flavonoids are a group of phenylpropanoic secondary metabolites known to influence cellular processes. They are of interest as nutrition additives due to their health-promoting effect (57). However, the knowledge of the effects of flavonoids on cellular processes in either animals or plants is still limited. The *Arabidopsis rol1-2* shoot phenotype, characterized by defects in cell growth and development, is induced by flavonols, a subgroup of flavonoids (30, 44), and thus represents a model to characterize the active flavonol(s) and their mode of action.

*Flavonol Glycosides Influence Plant Development*—The study presented here identifies 7-*O*-rhamnosylated flavonols as being important for inducing the growth defects in *rol1-2*. The enzyme UGT89C1, conjugating rhamnose onto flavonols (29), is encoded by a single gene in *Arabidopsis*, and mutations in *UGT89C1* were identified as suppressors of the *rol1-2* pheno-

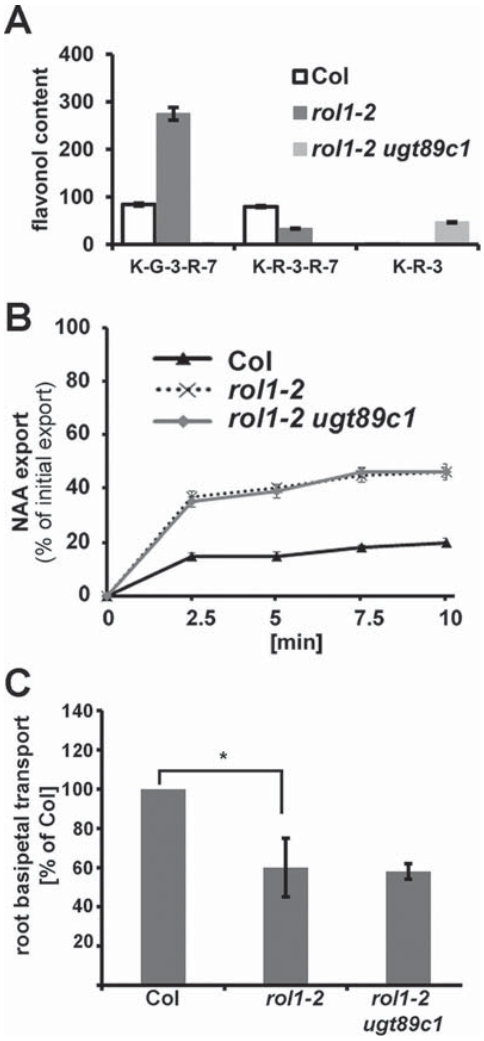


FIGURE 6. **Auxin transport in *rol1-2* plants is not affected by *ugt89c1*.** *A*, flavonol content analysis of protoplasts revealed the same effects of *rol1-2* and *ugt89c1* mutations as in intact plants. The area under each peak of the HPLC elution profile was used to determine flavonol content. *K-G-3-R-7*, kaempferol-3-*O*-glucoside-7-*O*-rhamnoside; *K-R-3*, kaempferol-3-*O*-rhamnoside. *B*, auxin (1-NAA) export from protoplasts is affected in *rol1-2* as compared with the wild type (*Col*) but not altered by the *ugt89c1* mutation. *C*, as compared with the wild type (*Col*), *rol1-2* and *rol1-2 ugt89c1* show similar, reduced basipetal IAA transport in roots. *Asterisks* indicate statistically significant differences (*t* test, $p < 0.05$). *Error bars* indicate mean ± S.E.

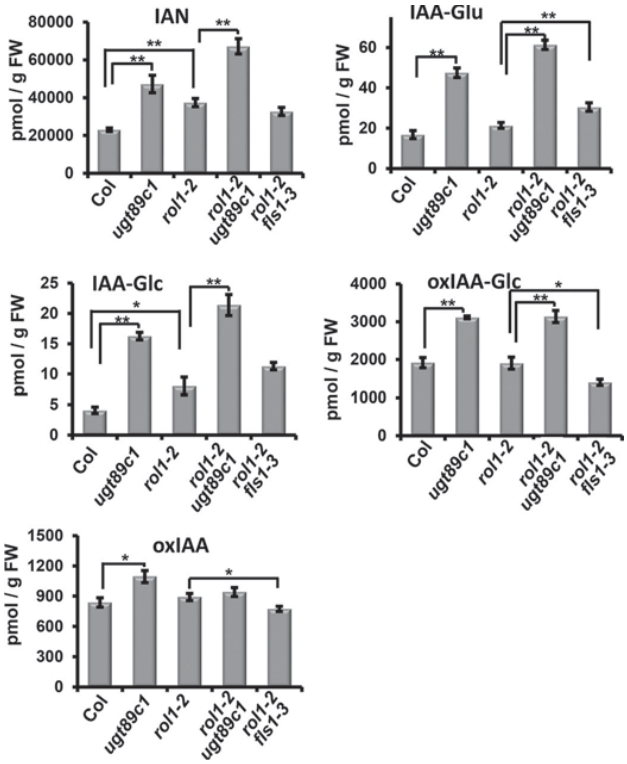


FIGURE 7. ***ugt89c1* affects levels of auxin precursor and metabolites.** Quantification of the auxin conjugates IAA-Glc and IAA-Glu, the auxin precursor IAN, and the degradation products oxIAA and oxIAA-Glc is shown for wild type (*Col*), *rol1-2*, and *ugt89c1* single mutants, and for *rol1-2 ugt89c1* and *rol1-2 fls1-3* double mutants. *FW*, fresh weight. Statistically significant differences (*t* test) with $p < 0.05$ and $p < 0.01$ are indicated with one and two asterisks, respectively. *Error bars* indicate mean ± S.E.

type. In seedling shoots, *UGT89C1* expression is limited to the adaxial side of cotyledons, emerging leaves, and trichomes. The same protein accumulation pattern has been found for ROL1/RHM1 and FLS1 (44), which explains the asymmetry in the flavonol-induced growth phenotype that is limited in cotyledons to the adaxial side. The co-localization is also in line with previous findings that genes involved in flavonoid biosynthesis (including *FLS1*, *RHM1*, and *UGT89C1*) are co-regulated (26). The data suggest that flavonol glycosides are the active flavonol species and that 7-*O*-rhamnosylated flavonols are involved in inducing the *rol1-2* phenotype. Evidence for an active role of glycosylated flavonols in plant development was also provided by the analysis of the 3-*O*-glucosyltransferase mutant *ugt78d2*, which shows a dwarfed growth phenotype. This mutant contains increased levels of 3-*O*-7-*O*-rhamnosylated flavonols, and mutating either the 3-*O*-rhamnosyltransferases or the 7-*O*-rhamnosyltransferases *ugt78d1* or *ugt89c1*, respectively, suppresses the dwarfed phenotype of *ugt78d2*. Hence, 3-*O*-7-*O*-rhamnosylated flavonols appear to induce the growth defects developed by the *ugt78d2* mutant (31). Both the *ugt78d2* and the *rol1-2* mutant phenotypes are also found in combination with the *tt7* mutation (30, 31) that prevents synthesis of quercetin and causes over-accumulation of kaempferol (Fig. 1). Thus, accumulation of the flavonol kaempferol appears to be sufficient to interfere with proper plant development. Similarly, the *tt7* single mutant was reported to have increased inhibition of auxin transport, again suggesting that kaempferol and its glycosylated forms are particularly active flavonols (13). The mechanism of flavonol-induced growth defects, however, is not the same in *rol1-2* and *ugt72d2*. First, in contrast to *ugt78d2*, there is a reduction of 3-*O*-7-*O*-rhamnosylated flavonols in *rol1-2* as compared with the wild type (30). Second, although introducing the *ugt89c1* mutation suppresses both *ugt78d2* and *rol1-2*, the introduction of the 3-*O*-rhamnosyl transferase mutation *ugt78d1* only suppresses *ugt72d2* but not *rol1-2* (30, 31). This raises the question as to how suppression of the *ugt78d2* and *rol1-2* mutant phenotypes is induced. Although the mechanism of suppression of the *ugt78d2* mutant by *ugt78d1* and *ugt89c1* has not yet been investigated (31), the

work presented here suggests that *ugt89c1* suppresses the *rol1-2* phenotype by modulating levels of the auxin precursor IAN and auxin metabolites rather than auxin transport.

*Flavonols Influence Auxin Homeostasis in Several Ways*—The suppression of *rol1-2* by *tt4*, *tt6*, and *fls1* (30, 44) fit the picture of flavonols being negative regulators of auxin transport (11). Indeed, interfering with flavonol biosynthesis in *rol1-2* by an *fls1* mutation reverts the modified auxin transport to wild-type levels and suppresses the *rol1-2* phenotype. In contrast to the *fls1* mutation, however, *ugt89c1* does not seem to interfere with auxin transport. As compared with the wild type, the *rol1-2* mutant shows an increase in auxin efflux in the protoplast assay but a decrease in auxin transport in the root basipetal transport. This discrepancy can be explained by the very different experimental setup in the assays. In the protoplast assay, primarily auxin efflux is measured from a non-polarized, cellular system after loading with radioactive auxin. In contrast, the root basipetal transport assay is performed on the entire root, so that the resulting value comes from the complex auxin transport machinery present in the root tissues. However, and importantly, the absence of any effect of the additional *ugt89c1* mutation is found in both systems. This unexpected finding can be explained by different flavonol species having different functions in terms of regulating auxin levels. Although some interfere with auxin transport, others seem to influence auxin metabolism. Kaempferols are important for the development of the *ugt78d2* and *rol1-2* phenotypes or for pollen development in maize (6, 30, 31), whereas other studies point at quercetin as an important flavonol (58, 59). In the *rol1-2 fls1* double mutant, all flavonols are absent, resulting in wild type-like auxin transport, whereas a defect in 7-*O*-rhamnosylation only interferes with auxin metabolism but not transport. An indication for multiple modes of action of flavonols was also obtained from analyzing the *rol1-2* shoot phenotype in more detail. In seedlings, a correlation between increased auxin levels and growth defects was observed. However, phenocopying the increased auxin levels in wild-type seedlings by exposing them to the auxin efflux inhibitor 1-naphthylphthalamic acid resulted in hyponastic cotyledons comparable with the *rol1-2* shoot phenotype, whereas the defects in pavement cell shape and trichome development were not observed (30). Hence, negatively regulating auxin transport is likely not the only way by which flavonols in the *rol1-2* mutant interfere with plant development. The results presented here suggest that UGT89C1 influences the activity of auxin biosynthesis and its conjugation/degradation. Although oxIAA and oxIAA-Glc are irreversible degradation intermediates, IAA-amino acid derivatives can mostly be converted back to active, free auxin (32–34, 60). Interfering with the activity of amidohydrolases that convert IAA-amino acid conjugates to free IAA affects free auxin levels and induces auxin-related phenotypes (61, 62). A mutation in *ugt89c1* causes an increase in the level of auxin precursor and auxin metabolites, the only exception being oxIAA accumulation in *rol1-2 ugt89c1* that is comparable with *rol1-2*. Both low-abundant (such as IAA-Glu or IAA-Glc) as well as highly abundant (such as the auxin precursor IAN or the auxin degradation product oxIAA-Glc) auxin derivatives are affected to a similar extent. However, the low-abundant IAA derivatives are reversible conjugates that contribute to the pool of all IAA metabolites only very little (<1%). The accumulation of the precursor IAN in the *ugt89c1* and *ugt89c1 rol1-2* mutants suggests that conversion of this molecule to free IAA is inhibited. On the other hand, according to the levels of oxIAA-Glc, irreversible oxidative degradation is increased in those mutants. Thus, UGT89C1 seems to influence the production as well as the degradation of auxin. Considering the large amount of the precursor IAN in the entire pool of auxin-related metabolites, the regulation of the last step of auxin biosynthesis appears to be a main target process of the activity of UGT89C1. At this point, it is not clear why these changes in the pool of auxin and its derivatives are not reflected in a clear change in the level of free auxin. Several repetitions of auxin measurements gave somewhat varying results with an increase in *rol1-2* as compared with the wild type, whereas *rol1-2 ugt89c1* reduced auxin levels again. However, the differences were not severalfold, did not fulfill the criteria of statistical significance, and therefore cannot be considered in the interpretation. Such variations may reflect fine-tuning of levels of free auxin as the only auxin-active form in relation to subtle variations in developmental stages of plant material used to analyze auxin content in biologically independent samples. It can be assumed that the level of free auxin is under strict control and may be modified only temporarily and locally by control mechanisms that are functional in the absence of UGT89C1. Blocking flavonol biosynthesis in the *rol1-2 fls1-3* mutant has comparatively little effect on the auxin derivatives. In three out of five auxin-related compounds, the changes induced by *fls1-3* are the opposite of those induced by *ugt89c1*, *i.e.* oxIAA-Glc is reduced in *rol1-2 fls1-3* as compared with *rol1-2*, but increased in *rol1-2 ugt89c1*. These data indicate again that changes in the flavonol glycosylation pattern do not have the same effect on auxin levels as blocking altogether flavonol synthesis. Presumably, the balance between different glycosylated forms of flavonols including 7-*O*-rhamnosylated flavonols is critical for auxin homeostasis, and thus, for proper plant development. The mechanism by which flavonols influence auxin conjugation and catabolism remains to be uncovered. The level of oxIAA has been shown to be increased in the flavonol-deficient *tt4* mutant and reduced in the flavonol overaccumulator *tt3*, an observation that is attributed to the reactive oxygen species-scavenging activity of flavonols (9, 42). However, mutations in *ugt89c1* do not affect the overall accumulation of flavonols. This suggests that different flavonol glycosides might have different reactive oxygen species-scavenging activities, possibly because of distinct subcellular localization. Alternatively, *ugt89c1* mutations might not influence auxin oxidation via modulated reactive oxygen species-scavenging activity. It has been shown that changing flavonol glycosylation affects gene expression (8), and thereby, genes involved in auxin metabolism might also be affected. Formally, it cannot be excluded that UGT89C1 has another biochemical activity in addition to its function as a rhamnosyltransferase. However, UGT89C1 appears to recruit specifically Rha as the substrate and flavonols as acceptor for the glycosyltransferase reaction, and its gene expression is tightly linked with other genes involved in flavonol biosynthesis (26, 29), making such a scenario less likely.

Together, the findings of this study reveal the effect of flavonol glycosides on auxin metabolic turnover. In addition to the known effect of flavonols on auxin transport, this represents a new mechanism for flavonols to influence auxin homeostasis.

*Author Contributions*—B. M. K., S. E., R. B., P. D., and M. G. performed experiments; L. B., E. Z., and C. R. designed experiments; and C. R. designed the project and wrote the paper.

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# 6. Multiple functions of the Arabidopsis flavonol synthase FLS1 in flavonol biosynthesis, transcriptional regulation, and cell growth

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## 6.1 Abstract

Enzymes with well-defined biochemical activities can sometimes have additional functions and show unexpected subcellular distribution. One such enzyme is the *Arabidopsis thaliana* flavonol synthase FLS1. We found that, besides catalyzing the conversion of dihydroflavonols to flavonols, the last step in flavonol biosynthesis, FLS1 also influences transcriptional activity and cell growth processes. We show that the additional functions of FLS1 strictly depend on its subcellular localization. While an *fls1* mutant shows changes in gene expression compared to the wild type, nuclear FLS1 is required to re-initiate a wild-type expression pattern. Overexpression of *FLS1* in root hairs results in increased root hair elongation, a process that requires nuclear FLS1. Although cytoplasmic FLS1 is sufficient for accumulation of flavonols, which then effectively diffuse into the nucleus, nuclear FLS1 is important for the observed enhanced cell growth. The different activities of FLS1 can be genetically separated, resulting in FLS1 variants that can synthesize flavonols but are impaired in the other processes. Together, these results suggest that a single enzyme can have several partly independent functions that might help to integrate input from the plant's metabolic state onto different processes controlling cell growth and development.

## 6.2 Introduction

Flavonoids represent a large class of plant secondary metabolites with thousands of distinct molecules (Draeger et al., 2015). Flavonoids are produced via the phenylpropanoid pathway and have been shown to be important for cell growth processes. Mutations interfering with flavonoid biosynthesis lead to defects in pollen or root hair development (Mo et al., 1992; Taylor and Grotewold, 2005). In *Arabidopsis thaliana*, flavonoids have been shown to negatively influence transport of the phytohormone auxin. They alter localization of the PIN auxin transporters, inhibit the auxin transport proteins ABCB1 and ABCB19, and reduce the activity of the PINOID kinase (Jacobs and Rubery, 1988; Brown et al., 2001; Buer and Muday, 2004; Peer et al., 2004; Bouchard et al., 2006; Henrichs et al., 2012). The ROS (reactive oxygen species)-scavenging activity of flavonoids has been shown to influence auxin homeostasis (Peer et al., 2013). These functions are mainly attributed to flavonols that represent a side branch of the flavonoid pathway. Flavonols are glycosylated, in Arabidopsis mainly by Glc and Rha, and the glycosylation profile influences their activity. Changing flavonol glycosylation alters auxin homeostasis, by changing auxin transport and/or turnover (Yin et al., 2014; Kuhn et al., 2016).

The Arabidopsis rhamnose synthase mutant *rol1-2* has a modified flavonol glycosylation profile that induces defects in seedling shoot development with hyponastic cotyledons, brick-shaped pavement cells, and misshaped trichomes. In addition, *rol1-2* mutants show reduced root and root hair elongation. Interfering with flavonol biosynthesis by mutations in the chalcone synthase *TT4* or the flavonol synthase *FLS1* suppresses the *rol1-2* shoot phenotypes resulting in wild-type like shoot. In contrast, the root phenotype of *rol1-2* is largely flavonol-independent (Diet et al., 2006; Ringli et al., 2008; Kuhn et al., 2011).

While the biosynthesis of flavonoids has been localized to the cytoplasmic surface of the ER, several enzymes of this pathway including the flavonol synthase FLS1 have been found to localize also to the nucleus (Saslowsky and Winkel-Shirley, 2001; Saslowsky et al., 2005). Excluding FLS1 from the nucleus by a NES nuclear exclusion peptide (Wen et al., 1995) still results in complementation of the *rol1-2 fls1* double mutant phenotype (Kuhn et al., 2011). Hence, the biological relevance of the

nuclear localization of FLS1 is unclear. Flavonols have been identified in nuclei of several species (Saslowsky et al., 2005; Polster et al., 2006; Feucht et al., 2011, 2015) and potential functions of nuclear flavonols include DNA protection from UV damage and binding of histone proteins, which might influence chromosomal architecture and transcriptional activity (Peer and Murphy, 2007; Pourcel et al., 2013). Whether the nucleus-localized enzymes produce nuclear flavonols has not been elucidated.

In this work, we have investigated the biological relevance of nuclear FLS1 in Arabidopsis. Mutant analyses and expression of different versions of *FLS1* indicate that nuclear FLS1 has a considerable impact on the gene expression profile in roots, for example by modulating expression of genes involved in flavonoid biosynthesis. Overexpression of *FLS1* in root epidermal cells, where it is usually expressed at low levels, strongly increased root hair length, an effect that is dependent on nuclear FLS1. Mutational analysis of FLS1 revealed that the flavonol synthase activity of FLS1 can be separated from the effect on cell elongation, demonstrating that FLS1 is an enzyme that produces flavonols but has a concomitant role in nuclear processes influencing transcriptional activity and cell growth.

## 6.3 Results

### FLS1 influences gene expression

In a first step, the influence of FLS1 on gene expression was investigated using Arabidopsis ATH1 GeneChip Affymetrix microarrays representing 28'421 genes. Root tissue of 10-days-old seedlings of *rol1-2* and the *rol1-2 fls1-3* double mutant was used. *fls1-3* is a nonsense mutation abolishing almost completely flavonol biosynthesis (Kuhn et al., 2011). All microarray experiments were performed using biological triplicates. A factor of change in expression of ≥2 and *P-value* < 0.01 were used as a filter and resulted in 435 genes, of which 312 and 123 genes were down- and upregulated, respectively, in *rol1-2 fls1-3* versus *rol1-2*. The genes affected belong to a large number of functional categories based on Gene ontology (GO)-analysis (Supplementary Figure 1). It was interesting to find that genes involved in flavonoid biosynthesis showed downregulation, indicative of a positive feedback loop of flavonols on expression of these genes. Those involved in epidermal cell

differentiation are also downregulated in *rol1-2 fls1-3*, which confirms the previously described importance of FLS1 for epidermal cell differentiation (Kuhn et al., 2011).

To validate the microarray data, RNA was isolated from roots of 10 days-old *rol1-2* and *rol1-2 fls1-3* seedlings and quantitative RT-PCR (qRT-PCR) was performed on a subselection of genes (Figure 1A). This analysis confirmed the reduction in expression of genes involved in flavonoid biosynthesis in *rol1-2 fls1-3* mutants compared with *rol1-2*. A comparable downregulation was found in the *fls1-3* single mutant compared to the wild type (Figure 1B).

Hence, the effect on gene expression is not only found in the *rol1-2* mutant background but is a genuine effect of *FLS1*.

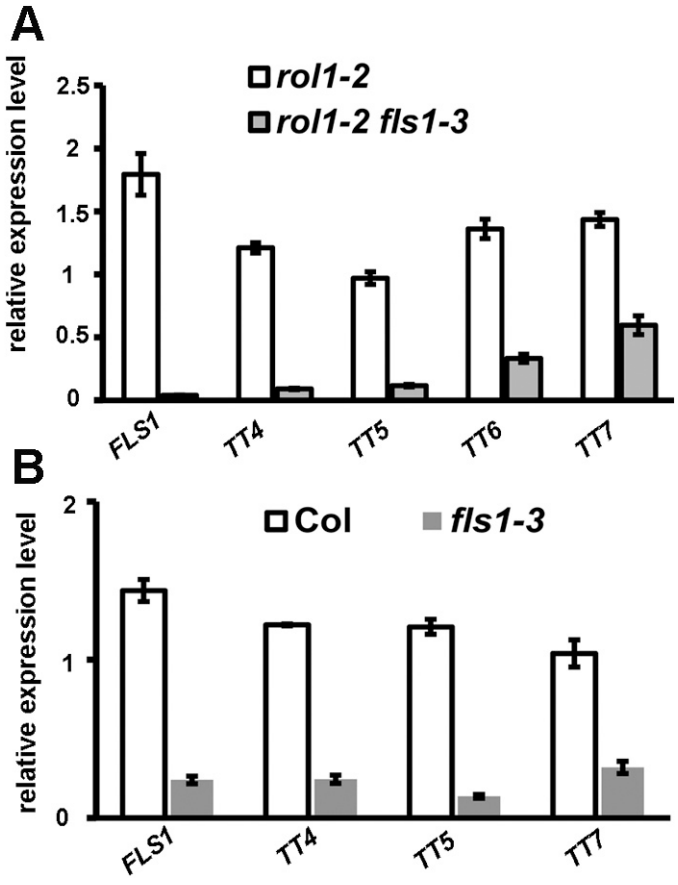



**Figure 1.** FLS1 **i**nfluences gene expression**.** Expression of genes of the flavonoid biosynthesis pathway was tested by qRT-PCR. RNA of roots of seven days-old seedlings was used as starting material. Each data point represents three technical replicates of each of three biological replicates, i.e. nine values. **(A)** Expression levels in *rol1-2* mutants are compared to those of the *rol1-2 fls1-3* double mutant. **(B)** Comparable changes in expression are found when comparing wild-type Columbia with the fls1-3 single mutant. Error bars represent standard deviations.

**Nuclear FLS1 modulates gene expression**

In the next step, we wanted to investigate whether nuclear FLS1 and/or flavonols, the product of FLS1 enzymatic activity, is important for the change in gene expression. To this end, *rol1-2 fls1-3* mutants were transformed with *GFP-FLS1* fusion constructs (Kuhn et al., 2011). In addition to *GFP-FLS1*, a *NES-GFP-FLS1* encoding an N-terminal Nuclear Exclusion Signal peptide (NES) previously shown to inhibit nuclear accumulation of proteins (Wen et al., 1995; Shen et al., 2007) and a *nes-GFP-FLS1* with a mutated, i.e. dysfunctional NES were expressed under the control of the *PIN2* promoter active in the epidermal- and cortex cell layers in the Arabidopsis root (Kleine-Vehn et al., 2008). The *PIN2* promoter was chosen since genes related to epidermal cell differentiation were among those showing modified expression in *rol1-2 fls1-3* compared with *rol1-2*, and *fls1-3* had been shown to affect epidermal cells in the shoot (Kuhn et al., 2011). The *PIN2:GFP-FLS1* construct indeed induced GFP fluorescence in regions of *PIN2* expression including root epidermal cells, which was stronger than fluorescence of a corresponding construct under the *FLS1* promoter (Figure 2A). Biosynthetic activity of the fusion protein was confirmed by the accumulation of flavonols in roots of two independent transgenic lines, while the parental *rol1-2 fls1-3* mutant only has minute amounts of flavonols (Figure 2B). The lower flavonol accumulation in the transgenic lines compared to the *rol1-2* mutant can be attributed to the *PIN2* promoter, which drives gene expression in a region that is more restricted to the apical area of the root compared to the endogenous *FLS1*. The GFP fluorescence pattern of the three fusion proteins showed that GFP-FLS1 and nes-GFP-FLS1 show strong accumulation in the nucleus, which is reduced with the NES-GFP-FLS1 (Figure 2C), confirming previous results that the NES peptide effectively reduced nuclear accumulation of FLS1 (Kuhn et al., 2011). In a complementary approach, *PIN2:NES-FLS1-GFP* and *PIN2:nes-FLS1-GFP* were transiently expressed in onion epidermal cells, which revealed the very same change in nuclear accumulation of GFP-FLS1 (Supplementary Figure 2).

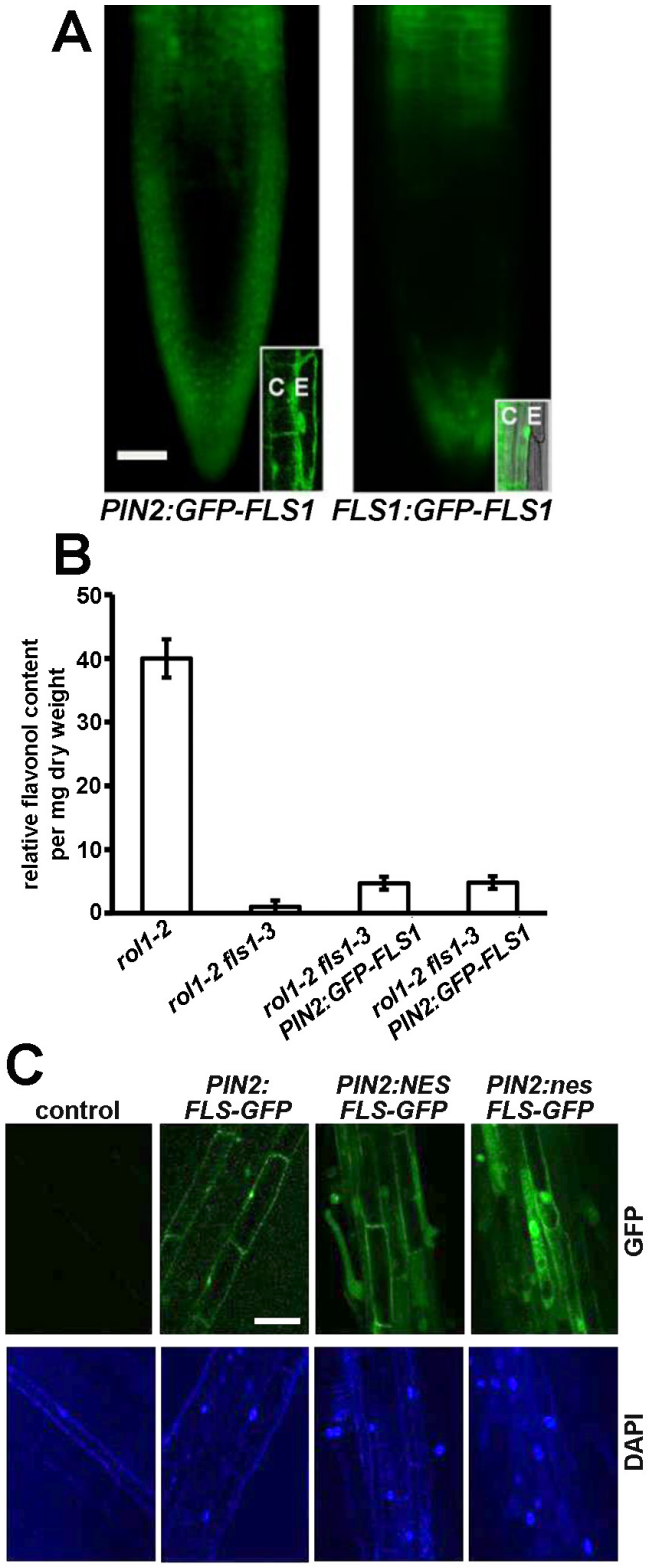



**Figure 2.** GFP-FLS1 under the control of the PIN2 promoter. (**A)** Compared with an *FLS1:GFP-FLS1* construct (right panel), the *PIN2:GFP-FLS1* construct (left panel) induces strong fluorescence in root epidermal cells of transgenic Arabidopsis seedlings. The inlets show cortical and epidermal cells at higher magnification. **(B)** Enzymatic function of GFP-FLS1 is reflected by the accumulation of flavonols in two independent *PIN2:GFP-FLS1* transgenic lines. The lower flavonol level in the transgenic lines is explained by the spatially limited activity of PIN2 in roots compared to the endogenous FLS1. **(C)** While GFP-FLS1 and nes-GFP-FLS1 accumulate in the nucleus; NES-GFP-FLS1 shows strongly reduced accumulation in the nucleus. The upper panel shows GFP fluorescence; the lower panel shows staining of nuclei with 4′,6-diamidino-2-phenylindole (DAPI). Bar = 40 µm (A); 10 µm (C).

To test whether the change in nuclear accumulation of GFP-FLS1 affects gene expression, the expression of the FBP-related genes was assessed by comparing the control *rol1-2 fls1-3* line and the *rol1-2 fls1-3* expressing either NES-FLS1-GFP or nes-FLS1-GFP. qRT-PCR analysis confirmed expression of the fusion constructs. Importantly, it revealed a moderate increase in expression in lines containing NES-GFP-FLS1 compared to the non-transgenic *rol1-2 fls1-3* and a stronger expression with nes-FLS1-GFP (Figure 3A). Hence, a correlation between abundance of FLS1 in the nucleus and induction of gene expression was observed.

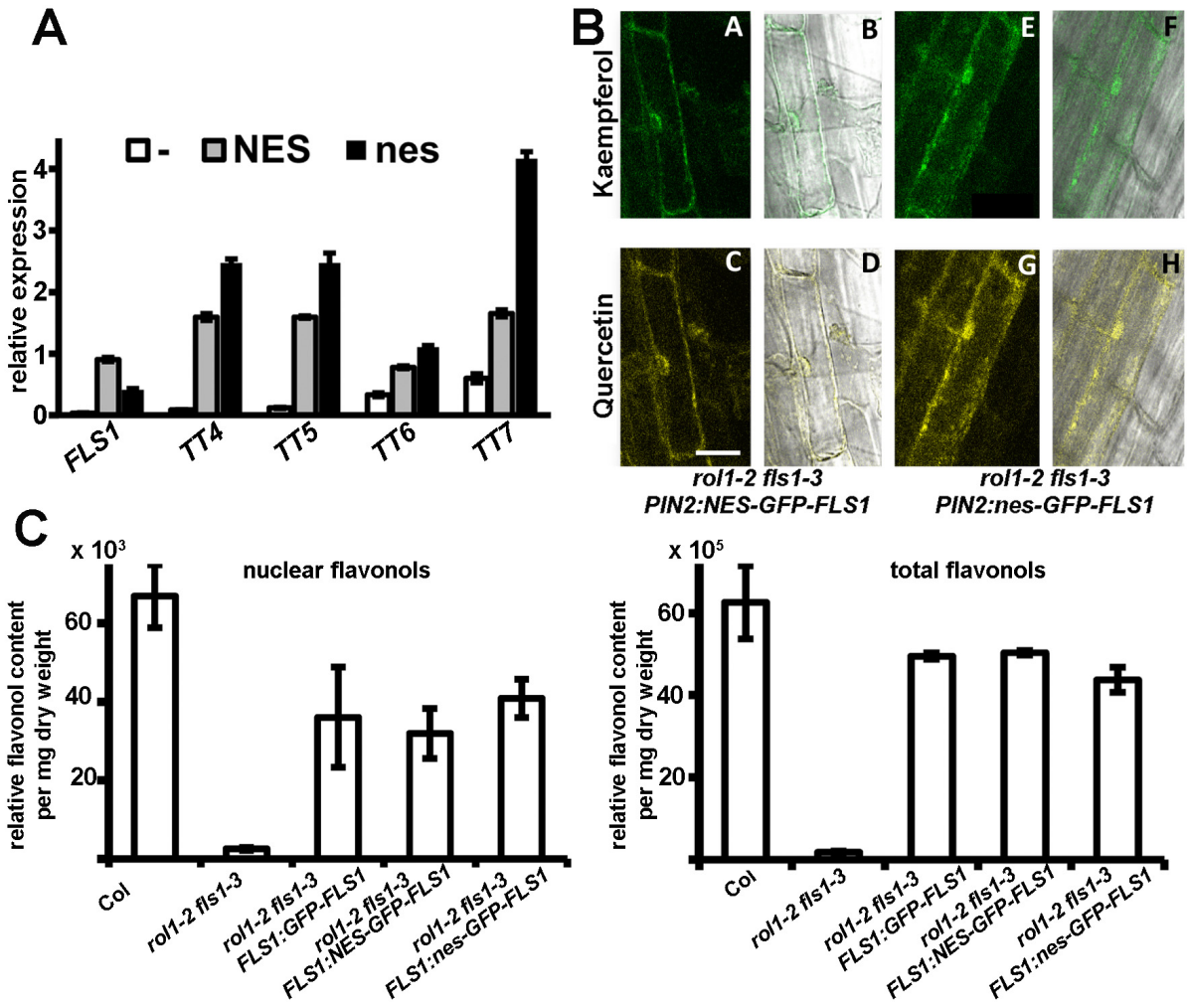



**Figure 3.** Nuclear FLS1 influences gene expression but not flavonol distribution. Nuclear and nucleus-excluded FLS1 is compared in terms of biosynthetic activity and gene expression. **(A)** Compared to *rol1-2 fls1-3*, nuclear-excluded NES-GFP-FLS1 and nuclear nes-GFP-FLS1 show increasing expression of genes of the flavonoid biosynthesis pathway. RNA of roots of seven days-old seedlings was used as starting material for qRT-PCR. Flavonol accumulation in the different lines is comparable as shown by diphenylboric acid 2- aminoethyl ester (DPBA) staining in situ **(B)** and **(C)** in flavonol total extracts (right) or nuclear extracts (left). Bar = 10 µm.

**Flavonol distribution is not dependent on FLS1 localization**

Next, it was investigated whether changing the spatial distribution of GFP-FLS1 also affects the accumulation of flavonols. To this end, Arabidopsis roots containing either NES-GFP-FLS1 or nes-FLS1-GFP were stained with the flavonoid-specific dye diphenylboricacid-2-aminoethyl ester (DPBA) that exhibits emission once bound to flavonols (Buer et al., 2007). Cytoplasmic and nuclear flavonol accumulation was observed in both lines, as seen by the green and yellow fluorescence emanated from DPBA-bound kaempferol and quercetin, respectively (Figure 3B). To confirm this finding, flavonols were extracted from either total tissue or from purified nuclei. For this analysis, *rol1-2 fls1-3* mutants transformed with the *GFP-FLS1* constructs under the *FLS1* promoter (Kuhn et al., 2011) were used, due to the higher flavonol levels compared with the lines containing the constructs with the *PIN2* promoter. Since the amount of nuclear flavonols represent only a very small fraction of the total extract, the latter can be considered as the cytoplasmic/vacuolar fraction of flavonols. Quantification of flavonols from nucleic and cytoplasmic/vacuolar fractions was performed using HPLC-MS (for details, see Methods) and the absence of the peaks in the *rol1-2 fls1-3* mutant was used as a control for proper identification of flavonols. As indicated by the *in situ* staining of flavonols by DPBA, all three transgene-encoded FLS1 variants lead to the accumulation of flavonols. No significant change in the absolute levels of flavonols in either the nuclear or the cytoplasmic/vacuolar fractions were found between the lines containing GFP-FLS1, NES-GFP-FLS1, and nes-FLS1-GFP (Figure 3C). Thus, the accumulation of nuclear flavonols does not depend on nuclear FLS1 and flavonols seem able to diffuse into nucleus.

**FLS1 suppresses *rol1-2* root and root hair elongation defect**

The *fls1-3* mutation has barely an effect on the short root and short root hair phenotype of *rol1-2* (Kuhn et al., 2011). However, when expressing *GFP-FLS1* under the *PIN2* promoter in *rol1-2 fls1-3*, the *rol1-2* root phenotypes were alleviated and transgenic plants developed wild type-like root hairs. To test whether the increased root hair elongation is flavonoid-dependent, a flavonoid-less *rol1-2 tt4* double mutant was transformed with *PIN2:GFP-FLS1*, which resulted in short root hairs (Figure 4A). In *rol1-2 fls1-3* mutants transformed with *PIN2:NES-GFP-FLS1*, long root hairs were

not observed (Figure 4A). Unexpectedly, expression of *nes-GFP-FLS1* also did not result in wild type-like root hair development. Hence, flavonoid accumulation is essential for the observed increase in root hair elongation. Since NES-GFP-FLS1 and nes-GFP-FLS1 cause flavonol accumulation, the biosynthetic activity of FLS1 is not sufficient to induce long root hairs. FLS1 seems to have additional functions that cause development of long root hairs, and these functions seem to be negatively influenced by the NES- and nes-peptides present in the corresponding fusion proteins.

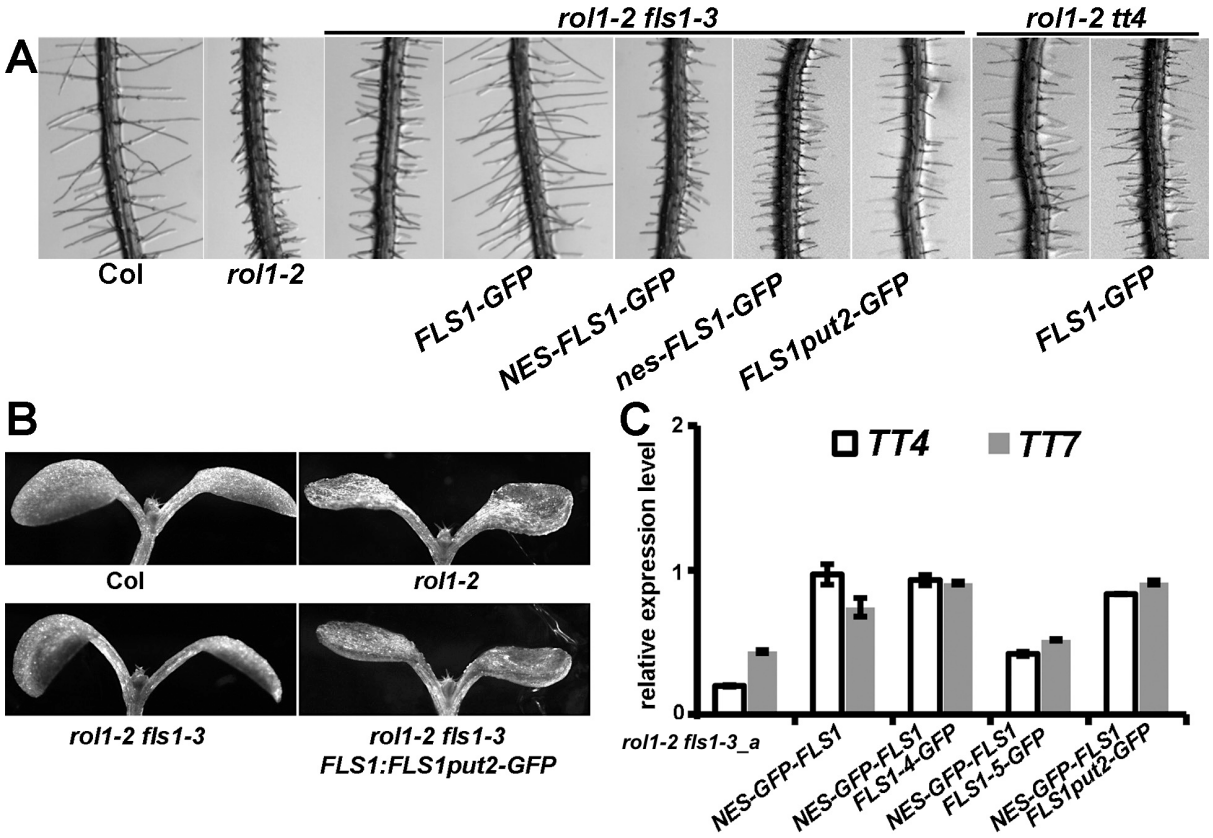



**Figure 4.** FLS1 induces changes in cell growth. **(A)** Compared to the short root hair-phenotype of *rol1-2* and *rol1-2 fls1-3*, *PIN2:GFP-FLS1* induces long root hairs. This effect is not observed in *NES-GFP-FLS1* or *nes-GFP-FLS1* expressing lines. Long root hair development is dependent on flavonoid biosynthesis, since it is not observed in the *rol1-2 tt4* double mutant background. **(B)** the *rol1-2* hyponastic growth phenotype of cotyledons is suppressed by the *fls1-3* mutation. The *FLS1Put2* allele under the *FLS1* promoter complements the *fls1-3* mutation. (**C)** The different *FLS1* mutant alleles expressed in the *PIN2:NES-GFP-FLS1* transgenic background do not further increase gene expression.

**Individual functions of FLS1 can be separated**

Different functions of the same protein might be separable by mutations that interfere with one but not all protein activities. Therefore, different mutations in FLS1 were tested for separation of the activities causing flavonol biosynthesis and changes in cell growth and gene expression. *fls1-4* and *fls1-5* were identified as suppressors of the *rol1-2* shoot phenotype and are therefore missense alleles interfering with flavonol biosynthesis (Kuhn et al., 2011). To investigate whether these FLS1 variants might still be able to induce long root hairs, *PIN2:FLS1-4-GFP* and *PIN2:FLS1-5-GFP* constructs were established. Since our data indicate that flavonols are necessary to induce long root hairs, these two constructs were transformed into plants containing *PIN2:NES-FLS1-GFP*, which produce flavonols but have short root hairs. The FLS1-4-GFP and FLS1-5-GFP proteins, however, had no effect on root hair length, suggesting that these proteins have lost any activity of FLS1.

In addition, the two new *FLS1* alleles *FLS1put1* and *FLS1put2* with mutations resulting in N223A and H75A substitutions, respectively, were produced. N223 and H75 are well conserved among several FLS enzymes of different plant species (Kuhn et al., 2011), implying a possible importance for FLS1 function. First, these new versions of FLS1 were tested for their enzymatic activity by complementing the *rol1-2 fls1-3* double mutant with *FLS1:FLS1put1-GFP* and *FLS1:FLS1put2-GFP*. While *FLS1put1* had no effect, *FLS1put2* caused complementation of *rol1-2 fls1-3*, resulting in a *rol1-2* shoot phenotype (Figure 4B). As expected, these complemented lines produced flavonols (data not shown), demonstrating that FLS1put2 is enzymatically active. However, when these two *FLS1* alleles were expressed under the *PIN2* promoter, neither FLS1put1-GFP nor FLS1put2-GFP induced long root hairs (Figure 4A). Thus, FLS1put2 represents a mutant FLS1 variant that is enzymatically active, i.e. accumulates flavonols, yet has lost the other activity/activities required for increased root hair elongation.

Finally, the effect of the mutant alleles of *FLS1* on gene expression was tested with *FLS1put2* which produces flavonols and the two alleles *FLS1-5* and *FLS1-4* that do not produce flavonols each of them containing *NES-GFP-FLS1*. Expression levels of *TT4* and *TT7* were tested since these were shown to be influenced by wild-type FLS1. qRT-PCR analysis revealed no increase in expression levels in by any of the

three alleles (Figure 4C). Hence, changes in gene expression and root hair elongation growth are not separated in these lines.

## 6.4 Discussion

In different plant species, growth and development of very different cell types and organs are modified by flavonoids, including flavonols (Mo et al., 1992; Taylor and Grotewold, 2005; Ringli et al., 2008; Maloney et al., 2014). Their role in influencing transport and metabolism of the phytohormone auxin is well-established (Brown et al., 2001; Buer and Muday, 2004; Peer et al., 2004; Kuhn et al., 2011; Yin et al., 2014; Kuhn et al., 2016). The data presented here demonstrates that the Arabidopsis flavonol synthase FLS1 has additional functions that go beyond the synthesis of flavonols and adds an additional layer of complexity by which the flavonoid biosynthesis pathway can influence cellular processes. This also provides hints as to how flavonoids can modify diverse processes such as the activity of signal transduction pathways or transcriptional activity (Peer and Murphy, 2006; Butelli et al., 2008; Rathee et al., 2009; Yamaguchi and Weitzmann, 2011; Avior et al., 2013).

### FLS1 is a protein with multiple functions

The shoot phenotype of the Arabidopsis *rol1-2* mutant is induced by flavonols and thus suppressed by mutations in *FLS1*, which largely abrogates flavonol biosynthesis (Ringli et al., 2008; Kuhn et al., 2011). The *fls1* mutation in *rol1-2 fls1-3* can be complemented with NES-GFP-FLS1 that is excluded from the nucleus, suggesting that cytoplasmic flavonol synthesis is sufficient for re-establishment of the *rol1-2* shoot phenotype. The nuclear flavonol content appears independent of whether FLS1 can enter or is excluded from the nucleus, indicating that the function of FLS1 in the nucleus is not primarily the synthesis of flavonols. Several enzymes of the flavonoid biosynthesis pathway including FLS1 have a dual localization in the cytoplasm and the nucleus (Saslowsky et al., 2005; Kuhn et al., 2011). This work reveals the potential of nuclear FLS1 to modify gene expression and cell growth as exemplified by increased root hair growth. The *fls1-3* mutation causes alteration in

expression of a large number of genes including those involved in flavonoid biosynthesis. Also others have found that alterations in flavonol accumulation affect other genes involved in flavonoid biosynthesis (Yin et al., 2012), revealing a positive feed-back loop of flavonols on the flavonoid pathway. However, our data show that the FLS1 protein is involved in this gene regulatory process and not solely the flavonols as the product of its enzymatic activity. The evidence for this assumption is that nuclear-localized nes-GFP-FLS1 is more effective in inducing gene expression than NES-GFP-FLS1, even though the two versions of FLS1 seem to produce comparable amounts of flavonols. At the other hand, nes-GFP-FLS1 does not increase root hair growth. It appears that the N-terminal nes peptide interferes with the currently elusive activity of FLS1 that causes increased cell elongation. While this activity is not flavonol biosynthesis, the presence of flavonols is still required, since in the flavonoid-less *rol1-2 tt4* double mutant background, GFP-FLS1 does not induce long root hairs. Thus, FLS1 integrates the information on flavonol abundance in its cell-elongating activity. The newly established FLS1put2 allele allowed to further separate functions of FLS1, as it induces flavonol biosynthesis but neither induces gene expression nor root hair elongation. The flavonol biosynthetic activity of FLS1 is independent of and separable from the other effects of FLS1, but there is currently no evidence that the inverse would be the case.

**The biological significance of dual localization of enzymes**

A number of metabolic enzymes was found to have dual localization in the cytoplasm and the nucleus in plants and other organisms. Some enzymes seem to sequester into the nucleus as a quickly available protein pool such as the yeast PI4P 5-kinase MSS4 (Audhya and Emr, 2003) or the mammalian Wiskott-Aldrich-Syndrome protein WASP which was initially identified as a regulator of peripheral actin polymerization but translocates to the nucleus in a manner that is regulated by its phosphorylation status (Suetsugu and Takenawa, 2003). There are, however, a number of examples of proteins with enzymatic activities in the cytoplasm that have a dual role in regulating transcription, translation, or RNA stability (Bhardwaj and Wilkinson, 2005). The glyceraldehyde-3-phosphate dehydrogenase GAPDH involved in glycolysis has been identified as an intrinsic component of a multiprotein coactivator complex

regulating gene expression in human cells (Zheng et al., 2003). In Drosophila, inosine monophosphate dehydrogenase can also directly bind DNA and attenuate histone gene expression. The DNA binding activity of this protein is independent of its enzymatic activity, demonstrating true dual functions of the same protein (Kozhevnikova et al., 2012). Arg5,6 is a mitochondrial protein from yeast involved in Arginine biosynthesis and was found to bind DNA and influence nuclear and mitochondrial gene expression (Hall et al., 2004). While it might be surprising at first that proteins can have very different functions in metabolism and gene expression, there is an obvious necessity of the metabolic state to impose itself on the regulatory state. So far, transcriptional regulation of genes encoding metabolic enzymes has been intensively studied, but the inverse regulatory impact is equally important (McKnight, 2010). In the case of the human GAPDH, this enzyme modifies the $NAD^+$:$NADH_2$ ratio and altering this redox status changes transcriptional activation through GAPDH (Zheng et al., 2003). Hence, the enzyme relates information on the redox state to the transcriptional machinery. Whether a metabolic/cellular status is represented by the activity of nuclear FLS1 protein remains to be shown. A potential link might be the levels of reactive oxygen species, which modify cell growth processes (Gapper and Dolan, 2006) and whose level is influenced by flavonols (Peer et al., 2013).

Several different flavonoid species have been detected in nuclei of diverse plant species (Saslowsky et al., 2005; Polster et al., 2006; Feucht et al., 2008). Their accumulation is developmentally regulated and can be modified under stress (Feucht et al., 2011). The biological relevance of this observation was so far controversial, and possible functions of flavonoids reach from shielding DNA from UV radiation to a direct effect on histone assembly, epigenetic modifications, and gene expression (Gilbert and Liu, 2010; Pollastri and Tattini, 2011; Draeger et al., 2015). Possibly, FLS1 is required for the proper function of the flavonols in the nucleus.

In summary, this work shows that the expression of *FLS1* results in several changes both on the level of cell development as well as gene expression. While some developmental processes are modified by the flavonols produced by FLS1, others require additional activities of this protein. The elucidation of the exact mode of

action of FLS1 in modifying gene expression needs further analysis. FLS1 might associate with flavonols that bind histones and thereby influence the epigenetic status as proposed by Feucht and coworkers (Feucht et al., 2008). Alternatively, FLS1 might directly bind DNA and thereby influence transcriptional activity. In silico analysis indicates that DNA binding motifs are present in the FLS1 protein, the biological significance of which needs to be analyzed. These results support the emerging picture of FLS1 being a protein with multiple functions, which are separable but not entirely independent. In this way, FLS1 can integrate information on the metabolic status such as abundance of flavonols and possibly other components and serve as a relay to adapt cell growth and development.

## 6.5 Material and methods

### Plant material

The *fls1-3* (nonsense), *fls1-4*, and *fls1-5* (both missense) alleles and the *rol1-2 tt4* line used in this study are described elsewhere (Kuhn et al., 2011). Growth of plants in sterile conditions, seeds were surface sterilized with 1% sodium hypochlorite, 0.03% Triton X -100, stratified 3-4 days at 4°C, and grown for 7 days on half-strength MS-medium containing 0.6% Phytagel (Sigma), 2% sucrose, 100 mg/l myo-inositol with a 16 h light / 8 h dark cycle at 22°C. For propagation of the plants, seedlings were transferred to soil and grown in growth chambers with a 16 h light / 8 h dark cycle at 22°C.

### DNA constructs, plant transformation, and molecular markers

The previously described *FLS1:FLS1-GFP:FLS1* construct (Kuhn et al., 2011) in the *pGEM-T* easy vector (Promega) was used to insert the point mutations by PCR-mediated mutagenesis to produce the different mutant versions of *FLS1*. For cloning of *PIN2:FLS1-GFP:FLS1* a KpnI site was introduced 5' adjacent to the start codon of the *FLS1:FLS1-GFP:FLS1* construct. The *PIN2* promoter was amplified with the primers *PIN2C_F2* CTCAAATGTATTAGGTTTGCTCAC and *PIN2_NGFP_R* GGTACCCATTTTGATTTACTTTTTCCGGC introducing a KpnI site at the 3' end of the promoter. This PCR fragment was cloned into pGEM-T easy (Promega),

sequenced, and a correct clone was used to replace the *FLS1* promoter with the restriction enzymes KpnI and SalI (cutting in pGEM-T easy), resulting in *PIN2:FLS1-GFP:FLS1*. To obtain the different mutant version of *FLS1* under the *PIN2* promoter, the BssHII and SpeI fragment of the different mutant *FLS1:FLS1-GFP:FLS1* variants, containing most of the *FLS1* coding sequence to the end of the terminator, was cloned into *PIN2:FLS1-GFP:FLS1* cut with the same enzymes. To produce *PIN2:NES-GFP-FLS1:FLS1* and *PIN2:nes-GFP-FLS1:FLS1*, a KpnI site was introduced 5' adjacent to the start codon of the corresponding constructs under the *FLS1* promoter (Kuhn et al., 2011) and the FLS1 promoter was replaced by the *PIN2* promoter as described above. For plant transformation, the different constructs were cloned into *pART27* (Gleave, 1992) by digestion with NotI.

**Microscopy**

Phenotypic screening analysis of all transgenic lines was performed using a binocular (Leica MZ125). GFP fluorescence analysis of root seedlings was performed with a laser scanning confocal microscope (TCS SP5; Leica Microsystems). GFP fluorescence was imaged at an excitation wavelength of 488 nm, and the emission signal was detected between 495 and 530 nm.

**DAPI and DPBA staining**

For DPBA staining, seven-days-old seedlings were grown and incubated for 7 min in saturated (0.25% w/v) DPBA with 0.02% (v/v) Triton X-100 and then washed in distilled water for 5 min. Samples were visualized using a Leica SP5 Confocal laser Scanning Microscope with an Ar-laser (458/488/514 nm) for GFP and a 543 nm HeNe-laser for YFP.

Seedlings were stained using 0.1 µg/mL 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) to visualize the cell nucleus by blue fluorescence and observed with a confocal laser-scanning microscope. The excitation wavelength was 405 nm and fluorescence collected in the range 425-475 nm.

**Flavonol content analysis**

The analysis of the flavonol accumulation profile was done as described in (Kuhn et al., 2016) with minor modifications. Seedlings were grown in a vertical orientation for 6 days as described. One hundred intact seedlings were taken and frozen in liquid

nitrogen, and lyophilized to determine the dry weight. Nuclei and cytosolic fractions were used to quantify nucleic and cytoplasmic flavonol content. The dried material, nuclei and cytosolic fractions were incubated in 500 µL of 80 % methanol overnight at 4° C and subsequently macerated with a pestle, followed by vigorous vortexing. After pelleting the cell debris by centrifugation, the supernatant was transferred to a fresh tube and evaporated in a Speed-Vac centrifuge, with the temperature being limited to a maximum of 42° C. After evaporation, the pellet was resuspended in 100 µL of fresh 80 % methanol and used for analysis. HPLC-ESI-MS and MS/MS experiments were performed on an Acquity UPLC (Waters) connected to a Bruker maXis high-resolution quadrupole time-of-flight mass spectrometer (Bruker Daltonics). An Acquity BEH C18 HPLC column (1.7 µm, 2.1x100 mm fitted with a 2x2 mm guard column) was used with a gradient of solvent A ($H_2O$, 0.1 % (v/v) HCOOH) and solvent B ($CH_3CN$, 0.1 % (v/v) HCOOH), at 0.45 mL flow rate and with a gradient from 5-30% B in 8 min and 30-99.5 % B in 1 min, washing at 99.5 % for 1 min and re-equilibration at 5 % B for 2 min.

**Quantitative real time PCR**

Total RNA was isolated from 10-days-old root seedlings grown in 1/2 x Murashige & Skoog (MS) medium with 16 h light / 8 h dark cycle at 22°C using Total RNA Isolation System KIT (Promega) following the manufacturer's instructions. cDNA was synthesized using 500 ng total RNA in 10ul reaction system using Bio-Rad iScript Kit according to the manufacturer's instructions and was used as a template for quantitative PCR amplification in a Bio-Rad CFX96™ Real-Time System. KAPA SYBER®FAST (KAPA BIOSYNTHESIS) was used as fluorescent reporter. A set of primers were designed to generate specific fragments ~ 200bp (see table in supplementary data). Elongation factor (EF-1α), Ubiquitin 10 (UBQ10) and GAPDH reference genes was generated to normalize the expression (see table in supplementary data. Biological triplicates and technical replicates were used for each experiment. Student's t test was used as a statistical tool, using two-tailed distribution and two-sample equal variance.

**Microarray analysis**

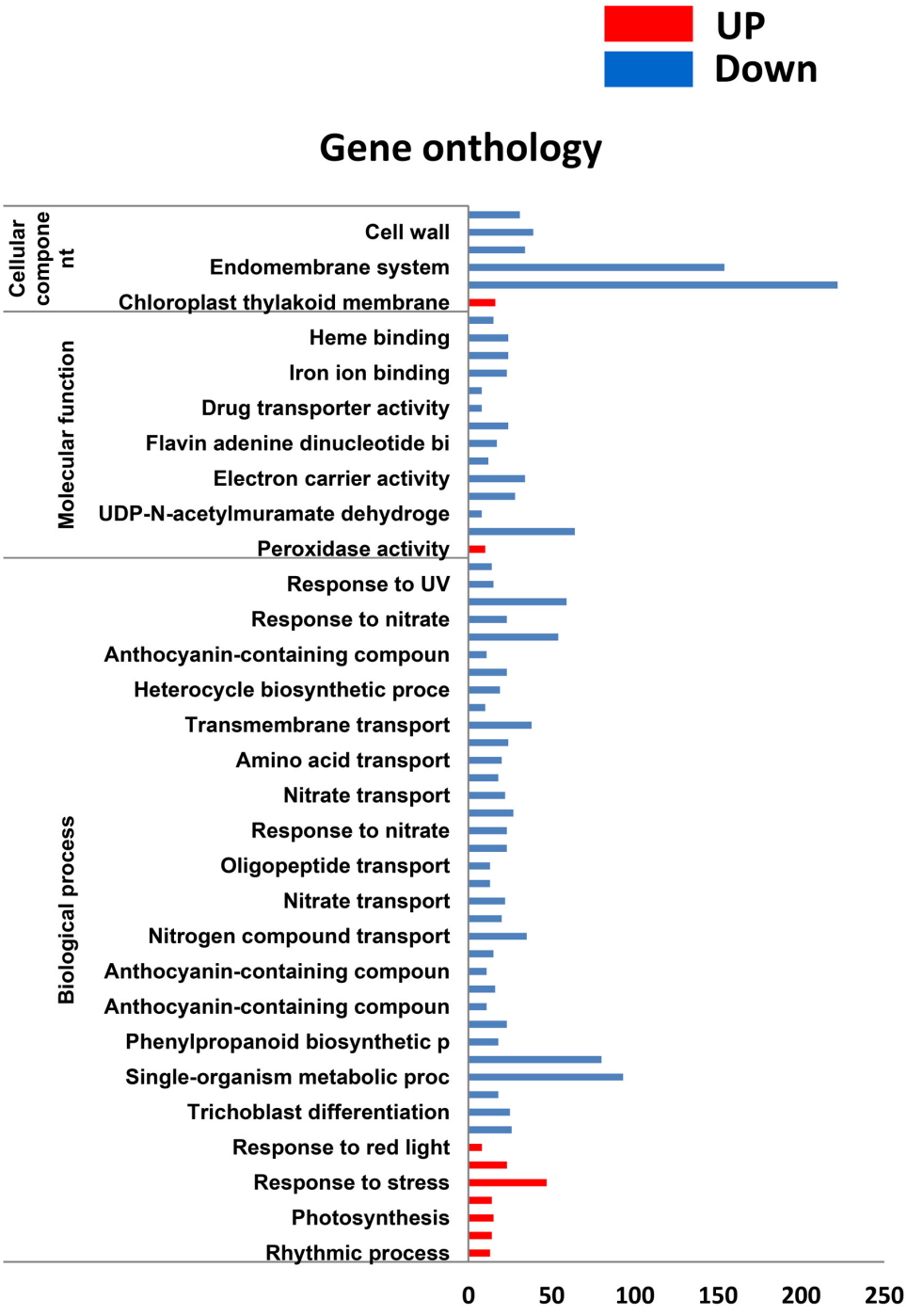
The quality of the isolated total RNA (100 ng) was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Delaware, USA) and a Bioanalyzer 2100 (Agilent,

Waldbronn, Germany). Only those samples with a 260 nm /280 nm ratio between 1.8–2.1 and a 28S/18S ratio within 1.5–2 were further processed. cDNA preparation was performed using the Ambion WT Expression Kit (P/N 4411973). Fragmentation and labeling was performed using Affymetrix GeneChip WT Terminal Labling Kit (P/N 901525). Biotin-labeled cDNA samples were mixed in 150 µl of WT Hybridization Cocktail (Affymetrix, P/N 901667) containing a Hybridization Controls and Control Oligonucleotide B2 (Affymetrix, P/N 900454). Samples were hybridized to GeneChip® Mouse Gene 1.1ST Array Strip (Affymetrix, P/N 901628) in the GeneAtlas Hybridization Station for 20 h at 48°C. Arrays were then washed using Affymetrix GeneAtlas Fluidics Station. An Affymetrix GeneAtlas Imaging Station was used to measure the fluorescent intensity emitted by the labeled target.

Gene expression profiling was performed using arrays of aragene10st-type from Affymetrix. The raw fluorescence intensity values were analyzed using the R packages Affy (Gautier et al., 2004) and Limma (Smyth, 2004) of Bioconductor (Gentleman et al., 2004). The gene expression data analysis consists of the following steps: 1. Between-array normalization, 2. Probe summary with the rma algorithm, 3. Fitting the data to a linear model and 4. Detection of differential gene expression. Quantile-normalization was applied to the log2-transformed intensity values as a method for between-array normalization, to ensure that the intensities had similar distributions across arrays (Irizarry et al., 2003). To find genes with significant expression changes between groups, empirical Bayes statistics were applied to the data by moderating the standard errors of the estimated values (Smyth, 2004). P-values were obtained from the moderated t-statistic and corrected for multiple testing with the Benjamini–Hochberg method (Benjamini and Hochberg, 1995). The P-value adjustment guarantees a smaller number of false positive findings by controlling the false discovery rate.

## 6.6 Supplementary Data

Supplementary Figure S1: Gene Ontology




**Supplementary Figure S1:** Gene ontology (GO)-based analysis. Genes differentially expressed between *rol1-2* and *rol1-2 fls1-3* using root of *Arabidopsis thaliana* are represented by their functional categories: biological process categorization, molecular function categorization, and cellular component categorization

## Supplementary Data

Table: Primes designed for the reference genes used in the qRT-PCR analysis

| Primers | 5’- 3’ Sequences (F/R) |
|---|---|
| Elongation factor _ (EF-1_) | F-TGAGCACGCTCTTCTTGCTTTCA<br>R-GGTGGTGGCATCCATCTTGTTACA |
| Ubiquitin 10 (UBQ10) | F-GGCCTTGTATAATCCCTGATGAATAAG<br>R-AAAGAGATAACAGGAACGGAAACATAGT |
| Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) | F-TTGGTGACAACAGGTCAAGCA<br>R-AAACTTGTCGCTCAATGCAATC |
| Flavonol Synthase 1 (FLS1) | F-CAGAGGTTGAGTAATGGGAGGT<br>R-GCTTGCGGTAACTGTAATCCTTG |
| Light-Harvesting Chlorophyll B-Binding Protein 3 | F-ACCCCCTTCGTGATGTTGTC<br>R-GTACTTCACTCTGTCAGGTCC |
| Peroxidase 5 | F-AGACCTTTCTTGCTGAAAAGACC<br>R-TGGGCAGTGAAAGTCGGAAG |
| Chalcone Synthase (TT4) | F- CATGACCGACCTCAAGGAGAAG<br>R-CGATGTCCTGTCTGGTGTCC |
| Chalcone Isomerase (TT5) | F-CGTTTGTACCGTCCGTCAAGTC<br>R-CTCCGTAGTTTTTCCCTTCCACT |
| Flavanone 3-hydroxylase (TT6) | F-ATCGTCTAGTCACCTCCAG<br>R-TCCTCCGTCACTTTCACCCA |
| Flavonol 3_-hydroxylase (TT7) | F-ACAGGAAGAGGTTGGAACGC<br>R-AGCCATCATTTCCGTCACCA- |
| MYB12 | F-GGGAAACAGGTGGTCACTAA<br>R-CTCGTTCTCCCAAGTCTGCG |


### 6.7 Acknowledgments

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# 7. *ugt89c1* mutation modifies the level of several hormones and induces a flavonol-independent root phenotype

## Abstract

Flavonols are secondary metabolites with diverse functions in plants. *In Arabidopsis thaliana,* they are subject to a glycosylation process that can alter their activity conferring to them their physiological properties. One of the UDP-dependent glycosyltransferases (UGTs) involved in the glycosylation process is the flavonol-specific 7-rhamnosyltransferase gene *UGT89C1*. The *Arabidopsis rol1-2* mutant is described to have an altered flavonol glycosylation profile that is modifying the shoot growth phenotype and auxin transport. Mutation in *UGT89C1* in a *rol1-2* background suppresses the shoot growth phenotype. Furthermore, the *ugt89c1* mutation was shown to increase auxin conjugates and catabolites, thereby altering auxin homeostasis, whereas auxin transport is not affected (Kuhn et al., 2011). Detailed analysis of *rol1-2* shows changes in several other phythormones, which are altered by the *ugt89c1* mutation, suggesting a role of flavonol glycosides in hormone distribution. Interestingly, UGT89C1 seems to have a flavonol-independent effect in plant development, raising the question whether the enzyme UGT89C1 is indeed specific for flavonols or rather has an additional, so far unknown function.

### 7.1 A *ugt89c1* mutation affects the aperture, length and density of stomata

The *rol1-2* plant has been shown to exhibit a stomatal phenotype compared to wild-type Columbia. The *rol1-2* cotyledons have fewer and larger stomata than those of wild-type plants (Ringli et al., 2008). Recently, mutations in*ugt89c1* were shown to suppress the *rol1-2* shoot phenotype (Kuhn et al., 2016). To assess whether a mutation in *ugt89c1* affects other plant growth aspects, measurement of stomatal aperture was performed in *ugt89c1* single mutant, *rol1-2 ugt89c1* double mutant, *rol1-2* single mutant, and wild-type Columbia lines. In this study we used the *ugt89c1-3* mutant allele for all experiments. Microscopic analysis revealed a reduction in the stomatal aperture (ratio length:width) in *rol1-2* mutant lines compared to wild-type Columbia (Figure 1, A-B). The *ugt89c1* mutation increased this stomatal aperture of *rol1-2* in the *rol1-2 ugt89c1* double mutant (Figure 1E). Furthermore, the single mutant *ugt89c1* increased strongly this aperture compared to wild-type Columbia (Figure 1E).

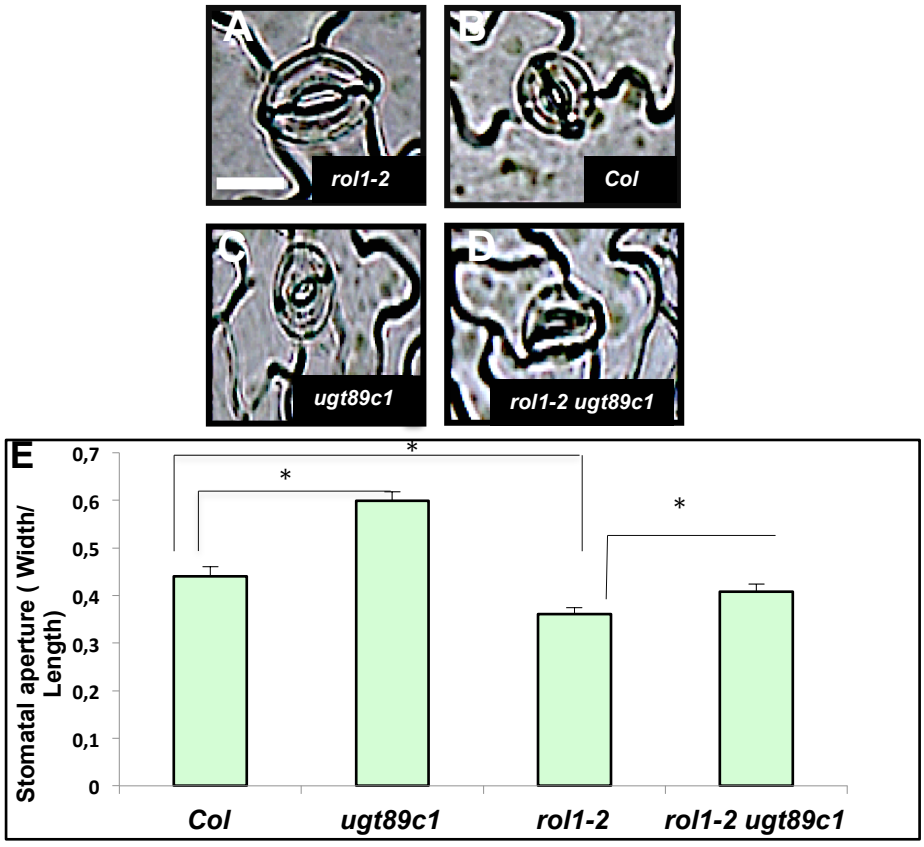


**Figure 1.** Stomatal aperture. **(A)-(D):** Rosette leaves were used for analysis of stomatal aperture. **(A):** *rol1-2* lines exhibit reduced stomatal aperture compare to wild-type Columbia. **(B):** wild-type Columbia shows a significant lower stomatal aperture compared to *ugt89c1*. **(C):** *ugt89c1* single mutant lines show the strongest stomatal aperture compared to all other lines. **(D):** *rol1-2 ugt89c1* double mutant lines reveal an increased stomatal aperture compared to *rol1-2* lines. **(E):** stomatal measurement

confirms the microscopic data. Asterisks refer to significant differences with a P<0.05. (**A-D):** *Bar=20 µm.*

The stomatal aperture was not the only process affected by the *ugt89c1* mutation. Other aspects of stomatal development were also investigated. The stomatal length was measured in the different lines. *rol1-2* lines showed bigger stomata compared to wild-type Columbia (45 ± 0,1 versus 42 ± 0,15 µm) as already shown in Ringli et al. (2008) (Figure 2A), whereas the *ugt89c1* mutant lines exhibited the smallest stomatal length (39 ± 0,1 µm) (Figure 2A). The effect of *ugt89c1* mutation in *rol1-2 ugt89c1* compared to *rol1-2* was visible but did not fulfill the criteria of statistical significance (t-test, P< 0.05). In addition, the stomatal density is affected in *rol1-2* lines where the density is two times less compared to wild-type Columbia (10 ± 2 versus 18 ± 5 per $mm^2$) (Figure 2B). Furthermore, the *ugt89c1* and *rol1-2 ugt89c1* mutant lines showed an even stonger reduction in stomatal density (7 ± 1 and 5 ± 1 per $mm^2$) compared to the wild-type and the *rol1-2* respectively (Figure 2B). Taken together, our results show that *ugt89c1* affects plant development in terms of stomatal aperture, length and density.

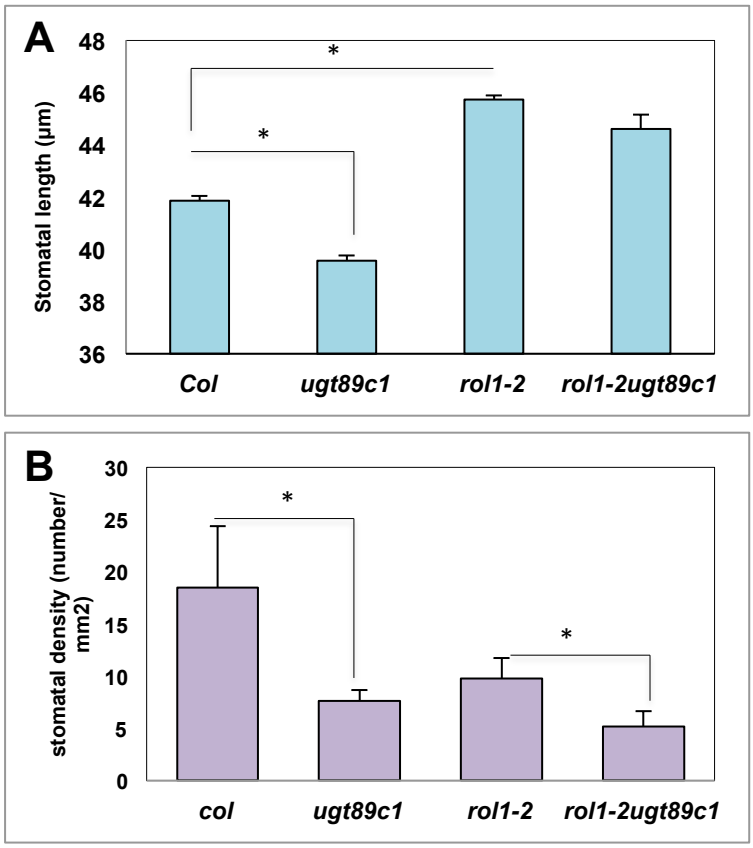


**Figure 2.** *rol1-2* and *ugt89c1* affect stomatal length and density**. (A):** stomatal length measurement (µm) of 6 week-old plants. t-test with *P< 0.01. (**B):** stomatal density (number/mm2) of 6 week-old plants. Asterisks represent significant difference using t.test *P< 0.01.

### 7.2 *ugt89c1* causes hypersensitivity to cytokinin treatment

Changing the accumulation of 7-rhamnosylated flavonols was shown to modulate the homeostasis of the plant hormone auxin (Kuhn et al., 2016). Plant physiological processes and development are under the control of various hormones which crosstalk in a complex manner e.g. auxin and cytokinin act antagonistically (Stepanova et al., 2011). To investigate whether the altered abundance of 7-rhamnosylated flavonols affects the response to other plant hormones, seedlings were grown in the presence of increasing concentrations of different hormones and root length was measured. Overall, all the lines have showed a decrease in root length while increasing the cytokinin concentration. Furthermore, the *ugt89c1* mutant showed a stronger decrease in root length compared to wild-type Columbia (Figure 3A). However, the *fls1-3* mutants were insensitive to cytokinin treatment since the roots showed always the same increased root length when increasing concentration (Figure 3A). The *fls1-3 ugt89c1* mutants show the same phenotype as *fls1-3* single mutant, revealing the implication of flavonols in cytokinin treatment. To explore whether the same effect of the *ugt89c1* mutation occurs in *rol1-2* background, the identical experiment was conducted with the corresponding mutant lines. Analysis of changes in root length revealed that all the lines exhibited a decrease in the root length when increasing the cytokinin concentration. However, *rol1-2 ugt89c1* mutants were more sensitive to cytokinin treatment and showed a stronger decrease in the root length (Figure 3B). In contrast, the lines carrying the mutation in *fls1-3* were significantly less sensitive to the treatment and exhibited an increased root length as without cytokinin treatment compared to the other lines (Figure 3B). Taken together, our results indicate that the *ugt89c1* mutation induces hypersensitivity to cytokinin treatment and this is flavonol-dependent. 7-rhamnosylated flavonols appear to interfere with Cytokinin-induced processes and affect root growth and development.

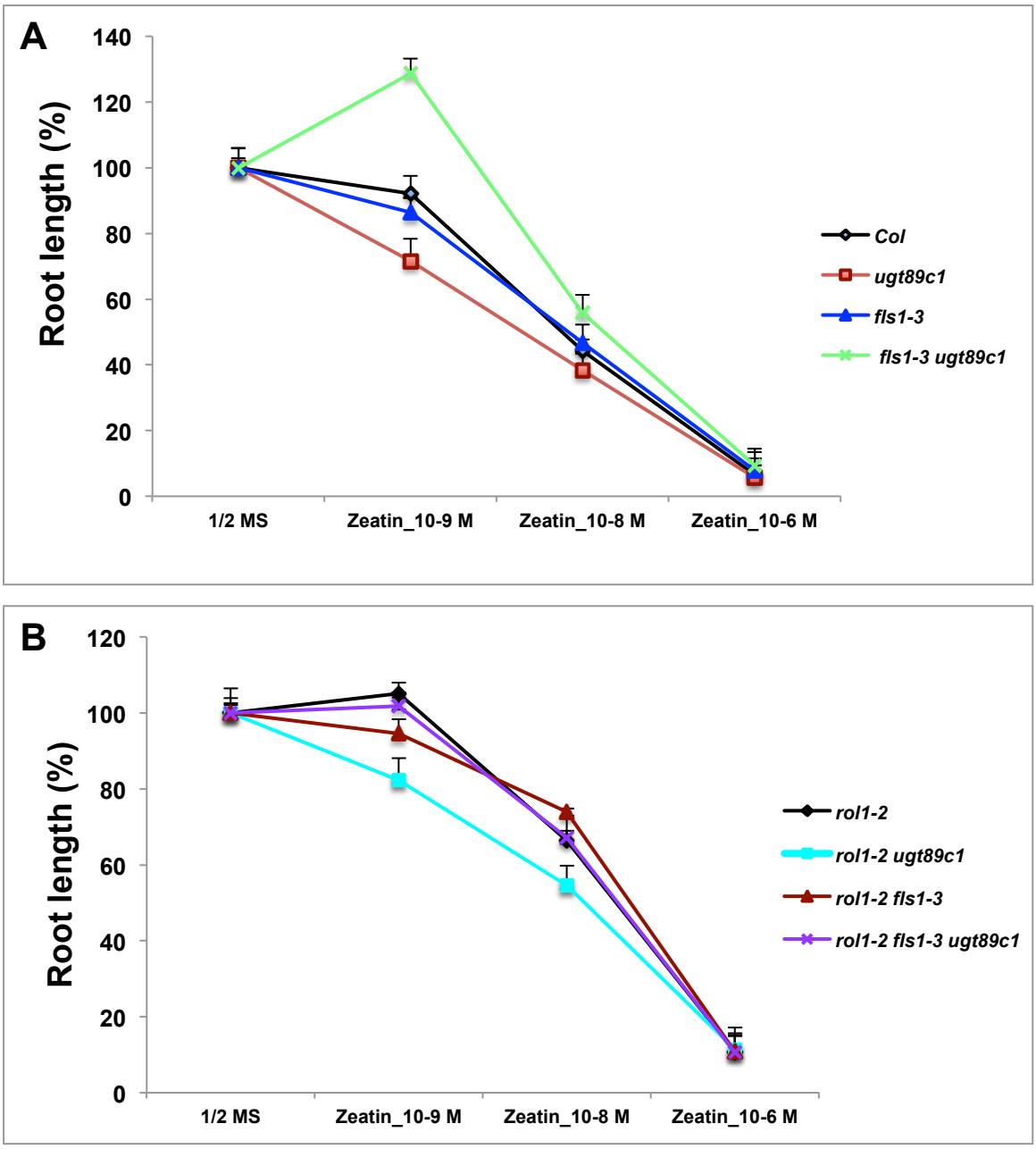


**Figure. 3**. The *ugt89c1* mutation alters the response of seedlings under cytokinin treatment. **(A)-(B):** root length measurement of 8 days old seedlings grown on half-strength MS with increasing zeatin concentrations. Length is indicated in % on control medium. N= 14-26 seedlings.

### 7.3 *ugt89c1* mutation mitigates the level of several hormones

Since changes in 7-rhamnosylated flavonols seem to interfere with the response to the plant hormone cytokinin, it was worth verifying whether these compounds also affect other hormones.

Therefore, the levels of different hormones were checked and measured in different lines carrying *ugt89c1* and *fls1-3* mutations. Here, the analysis revealed a significant increase of salicylic acid, jasmonic acid and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) in the *rol1-2* single mutant lines compared to wild-type Columbia (Figure 4, B-D). However, while the level of abscisic acid seems to follow the same pattern, the values between wild-type Columbia and *rol1-2* did not fulfil the criteria for significance (t-test P<0.05) (Figure 4A). This effect is alleviated in both *rol1-2 fls1-3* and *rol1-2 ugt89c1* double mutant lines (Figure 4, A-D). Taken together, our results indicate that flavonols in general and the 7-rhamnosylated flavonols in particular can modulate hormone levels. However, the hormonal cross talk is complicated and the mechanism by which flavonols can affect hormones and at which level remains to be elucidated.

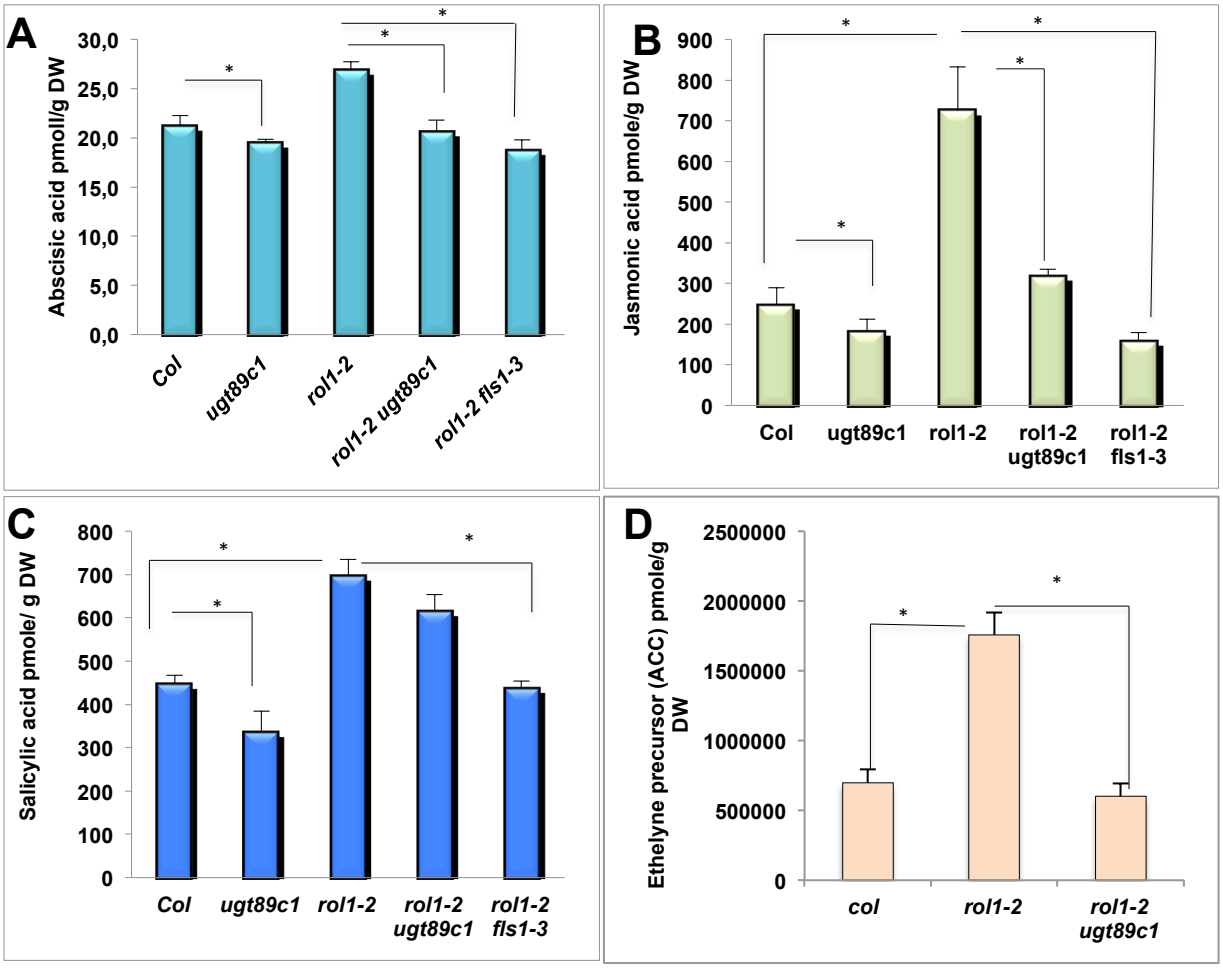


**Figure 4.** Flavonols modify level of several hormones. 10-day-old plants were used for hormone measurement. **(A):** Abscisic acid. **(B):** Jasmonic acid. **(C):** Salicylic acid**. (D):** Ethylene precursor (ACC), data on *rol1-2 fls1-3* were not obtained. t.test* P<0.05.

## 7.4 *ugt89c1* induces a flavonol-independent root hair phenotype

It has been shown that the mutation in *UGT89C1*, coding for a 7-rhamnosyltransferase reverts the cell shape formation defects observed in *rol1-2* as well as the hyponastic cotyledons to wild type-like phenotype (Kuhn et al., 2016). The lack of 7-rhamnosylated flavonols in *rol1-2 ugt89c1* suggests an important role of these compounds in inducing the *rol1-2* shoot phenotype and modifying auxin homeostasis (Kuhn et al., 2016). To investigate whether the *ugt89c1* mutation has also flavonol-independent effects on plant development, the *rol1-2 fls1-3* mutant plants lacking flavonols were compared to the triple mutant plants *rol1-2 fls1-3 ugt89c1.* The analysis revealed a bulging phenotype at the base of the root hairs of the triple mutant compared to the double mutant (Figure 3). Complementation of the *rol1-2 fls1-3 ugt89c1* triple mutant with *UGT89C1: UGT89C1* resulted in the suppression of the root hair bulging phenotype (Figure 5E), confirming that the *ugt89c1* mutation induces the root hair-bulging phenotype. This result demonstrates that the *ugt89c1* mutation has an effect on cell growth and development, which is independent of flavonols.

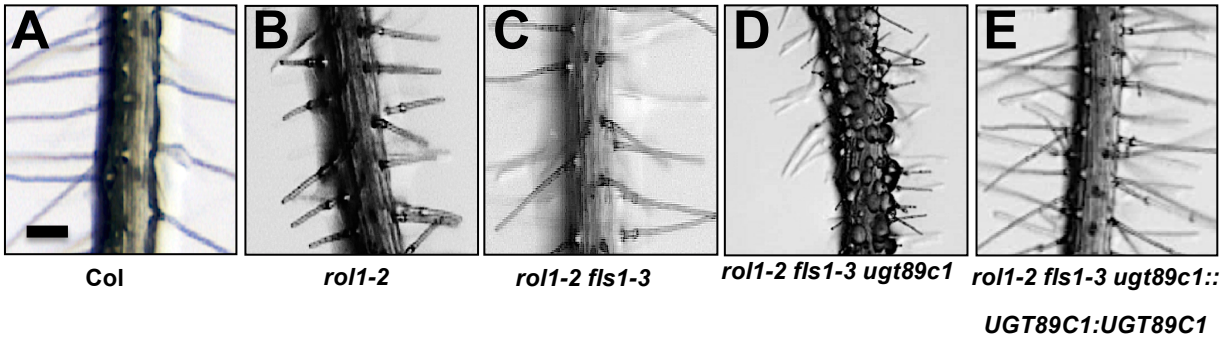


**Figure 5.** *ugt89c1* has a flavonol-independent effect on root hair development. Plants were grown for seven days on half-strength MS plates in a vertical orientation. **(A):** Wild-type Columbia plants. (**B):** *rol1-2* single mutant plants. **C:** *rol1-2 fls1-3* double mutant plants. **(D):** Triple mutant plants *rol1-2 fls1-3 ugt89c1* exhibiting the bulging phenotype*. **(E):*** Complementation experiment of the triple mutant lines *rol1-2 fls1-3 ugt89c1* with a *UGT89C1: UGT89C1* construct results in the suppression of the root-hair bulging phenotype. *Bar=500 µm.*

# 8. *In vivo* negative modulation of PID kinase by flavonols in the hypocotyl

## Abstract

Phototropism is one of the fastest and clearest visible adaptation responses of plants to environmental variations. This process enhances plant shoots to grow towards light and roots to evade it. It includes light perception and asymmetric distribution of the phytohormone auxin. The hypocotyl of *Arabidopsis thaliana*, which is characterized by a simple morphology and high responsiveness to growth factors and external stimuli, provides an excellent model system to study the effect of signals on physiological mechanism of cell elongation. The Ser/Thr protein kinase PINOID (PID) is implicated in the modulation of auxin efflux transporter PIN-FORMED (PIN) proteins through modification of their phosphorylation status. Flavonols, a subgroup of flavonoids, have been shown to inhibit auxin efflux in hypocotyl, proposing a specific flavonol function as transport inhibitor in hypocotyl. In this chapter, I show experiments that indicate that flavonols interact with PID and thereby modify hypocotyl growth and phototropism.

## 8.1 Flavonols affect hypocotyl growth

In order to investigate developmental growth defects induced by flavonols, seedlings with *rol1-2* and/or *fls1-3* mutations were grown vertically and hypocotyl length was measured. The *fls1-3* mutation was shown to convert the *rol1-2* shoot phenotype to a wild type-like phenotype (Kuhn et al., 2011), and this mutation was also able to alleviate significantly the short hypocotyl phenotype of the *rol1-2* mutant (Figure 6, B, C and E). This suggests the involvement of flavonols in the hypocotyl development of *rol1-2*. To test whether this phenotype in the hypocotyl is an accurate effect of the *fls1-3* mutation or only specifically observed in the *rol1-2* mutant background, hypocotyl length was assessed in wild-type Columbia versus *fls1-3* mutant. As shown in Figure 6A and 6D, an increased hypocotyl length is observed in the *fls1-3* mutants compared to wild-type Columbia. Hence, the effect of flavonols on hypocotyl growth is not only found in the *rol1-2* mutant background but is relevant also in the wild-type background (Figure 6E). Taken together, this data shows that flavonols, in particular those accumulating in *rol1-2*, affect the elongation of the hypocotyl.

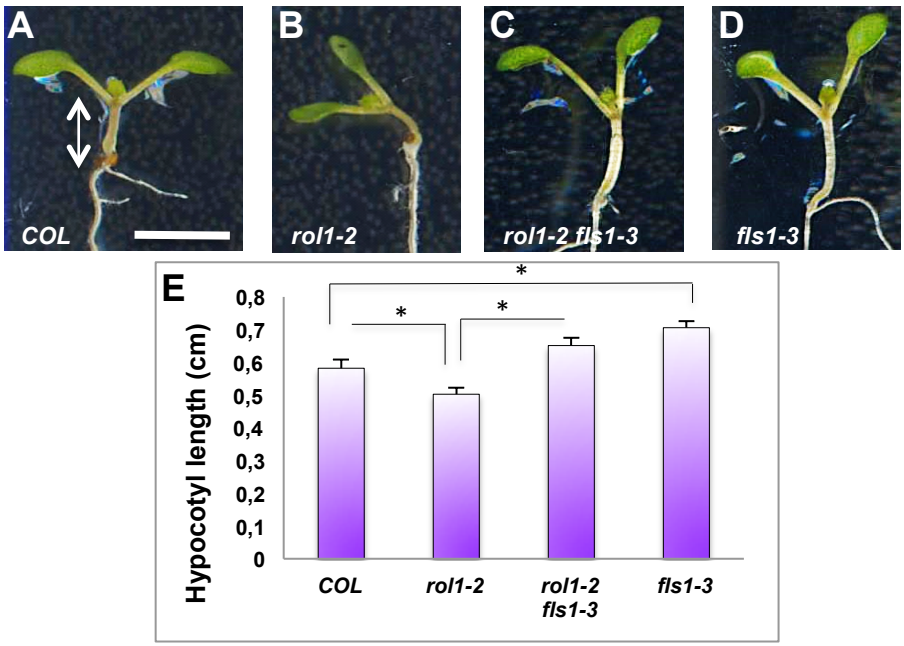


**Figure 6.** Hypocotyl elongation is modified by flavonols. 7 day-old seedlings were grown on half strength-MS medium in a vertical orientation and hypocotyl length was measured. **(A):** Wild-type Columbia. **(B):** *rol1-2* seedlings display reduced hypocotyl growth compared to wild-type. (**C):** *rol1-2 fls1-3* double mutant exhibits a longer hypocotyl compared to *rol1-2* seedlings. **(D):** *fls1-3* seedlings show an increased hypocotyl length compared to wild-type. (**E):** Hypocotyl measurement of the different lines using at least 24 seedlings per measurement. t-test *P<0.05. The arrow refers to the hypocotyl. *Bar=500 µm.*

## 8.2 PINOID induces hypocotyl bending and this effect is enhanced by the absence of flavonols

The *in vivo* analyses in the root have shown that flavonols negatively affect PID activity during the gravitropism process in *rol1-2* background (Kuhn et al., 2016b). Here the question was addressed whether a similar effect by flavonols can be detected in a different level of the plant. Therefore, hypocotyl curvature measurement was taken as an approach to answer this question. First, the effect of *PID* under the *FLS1* promoter was tested in *rol1-2* and the flavonol deficient mutant *rol1-2 fls1-3*. As shown in the Figure 2A and 2B, the expression of *PID* in the *rol1-2* increased significantly the hypocotyl bending. This effect is stronger in the absence of flavonols, suggesting a negative modulation of PID activity by these secondary metabolites during the hypocotyl phototropism process.

In a next step, it was necessary to investigate whether this effect is specific to *rol1-2* or rather a general effect. Wild-type Columbia and *fls1-3* were transformed with the *FLS1:PID* construct. Analysis revealed that the hypocotyl bending was increased in the *fls1-3* compared to wild-type, confirming a role of flavonols in modulating hypocotyl phototropism. Expressing *PID* in these lines further increased the hypocotyl curvature. Importantly, here also, this effect of the PID kinase was enhanced by the absence of flavonols (Figure 2C). Taken together, our data suggest an *in vivo* negative modulation of the PID kinase by flavonols in the hypocotyl and thus confirm the comparable finding in root gravitropism experiments as described in Chapter 4.

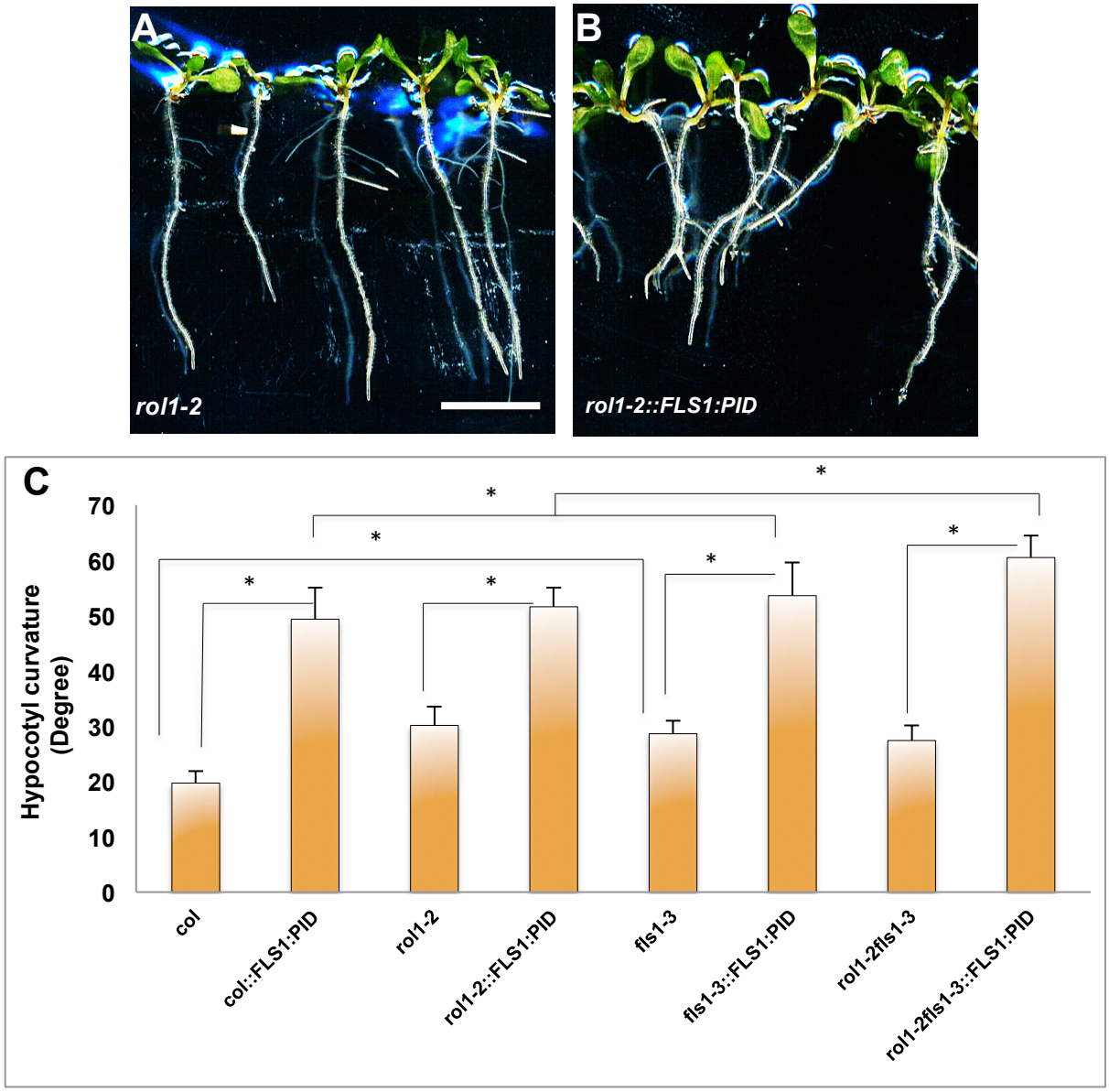


**Figure 2.** PINOID induces hypocotyl bending and this effect is enhanced in the absence of flavonols. **(A)-(B):** Representative pictures of seedlings used for analysis. *rol1-2* single mutants show less hypocotyl bending whereas *rol1-2::FLS1:PID* seedlings exhibit a stronger hypocotyl bending in random directions. **(C):** Measurement of hypocotyl curvature of 10 day-old seedlings grown under unilateral white-light illumination (2 µmol $m^{-2}s^{-1}$) and the average curvatures were calculated from at least 26 seedlings. t-test *P<0.05. *Bar=1cm.*

# 9. General discussion

## 9.1 General discussion

Since many decades, flavonoids and their subgroup flavonols were subject to an increasing scientific interest because of their versatility and multi-functionality in plants. These secondary metabolites are known to have different cellular targets, and thereby regulate multiple cellular pathways. To gain more insights into novel modes of action of flavonols in modulating plants, using the *rol1-2* mutant as a tool, my thesis aimed to answer the following questions:

1. How does the modified flavonol profile in *rol1-2* affect auxin homeostasis and therefore plant growth?
2. Do flavonols influence plant development through modulating PID activity?
3. What is the biological significance of nuclear FLS1?

**Negative regulation of PID kinase by flavonols in the hypocotyl**

The study made by Kuhn et al. (2016) demonstrates that flavonols in *rol1-2* induce the alteration of the kinase/phosphatase activity equilibrium. This occurs through the modification of the phosphorylation status of PIN2 by altering PID kinase activity by the flavonols. Inhibiting the phosphatase PP2A activity by the *rcn1-3* mutation brings back to balance the kinase/phosphatase activity. Since that the inhibitory effect of flavonols on PID activity *in vitro* was already shown by Henrichs et al. (2012), my contribution in this work consisted in providing *in vivo* evidence of the negative effect of flavonols on PID activity. Using transgenic lines expressing *FLS1:PID*, I have investigated the negative effect of flavonols on PID activity *in vivo* through examinating PID-induced agravitropism as described by Benjamins et al (2001). This finding shows that PID has a stronger effect in the absence of flavonols. Hence, it was interesting to assess whether the same effect of flavonols on PID kinase happens in other tissues such as hypocotyl during the phototropism. Phototropism is an adaptation response during which plants grow relative to the incoming light (Whippo and Hangarter, 2006) and which involves several responses, including light perception and asymmetric distribution of the plant hormone auxin (Esmon et al., 2006; Fuchs et al., 2003; Li et al., 2005; Liscum et al., 2014; Sakai et al., 2001).

These adaptive growth regulations permit the boost of organ adjustment for better light perception at the shoot level, and for better water and nutrient uptake at the root level (Rakusová et al., 2011). The analysis of the hypocotyl bending using *rol1-2* plants transformed with an *FLS1:PID* construct revealed that expressing *PID* increases the hypocotyl curvature. Moreover, this bending effect significantly increased in the absence of flavonols, providing additional *in vivo* evidence for the negative modulation of PID activity by these secondary metabolites. This effect is not specific to *rol1-2* mutant because expressing *PID* in wild-type Colombia increases the hypocotyl bending and this effect is stronger in the absence of flavonols as shown in the case of *fls1-3::FLS1:PID* compared to *Col::FLS1:PID* plants. In plants, Auxin efflux carrier PIN-FORMED (PIN) proteins, such as PIN3, are thought to have central roles in regulating asymmetrical auxin translocation during tropic responses, including gravitropism and phototropism (Haga and Sakai, 2012). A negative regulation of PID was shown to lead to polarization of these PIN3 efflux carriers (Ding et al., 2011; Sorefan et al., 2009) and induce an auxin flow redirection to the shaded side of the hypocotyl. Therefore, auxin enhances growth and induces hypocotyl bending toward the light (Ding et al., 2011). It would be an interesting experiment to investigate the localization of PIN3 proteins. Possibly, PIN3 shows an altered localization in *rol1-2* and/or *rol1-2*::*FLS1:PID*, which could explain the reduced or increased bending in these lines, respectively. Further, flavonols seems to interfere with the hypocotyl growth. Our data here shows an increased hypocotyl length induced by *fls1-3* mutation in the *rol1-2* background compared to the *rol1-2* single mutant. The same result was obtained when comparing wild-type Columbia to the single mutant *fls1-3*, suggesting a genuine effect of this mutation on hypocotyl growth. Similarly, increased hypocotyl length was demonstrated in *transparent testa 6 (tt6*) mutants which are defective in the *FLAVANONE 3-HYDROXYLASE* (F3H) (Buer and Djordjevic, 2009). The increased hypocotyl length of *rol1-2 fls1-3* compared to *rol1-2 or fls1-3* versus wild-type might be explained by the inhibition of auxin transport in the hypocotyl by flavonols. A link between the elongation of hypocotyl and auxin transport was reported. A treatment with 1-naphthylphthalamic acid (NPA), an auxin transport inhibitor, has led to the inhibition of hypocotyl elongation (Jensen et al., 1998). Therefore auxin transport appears to be crucial for hypocotyl elongation. It would be interesting to investigate the auxin transport status in the hypocotyl level in both *rol1-2 fls1-3* compared to *rol1-2* and *fls1-3* versus wild-

type.

### 7-*O*-rhamnosylated flavonols do not solely modulate auxin homeostasis but also affect other hormone levels

The study presented here suggests that flavonol glycosides are active flavonol species and that 7-*O*-rhamnosylated flavonols are implicated in inducing the *rol1-2* phenotype (Kuhn et al., 2016) While an *fls1* mutation suppresses *rol1-2* by changing auxin transport back to wild-type levels (Kuhn et al., 2011), this work indicates that *ugt89c1* suppresses the *rol1-2* phenotype by modulating levels of the auxin precursor IAN and auxin catabolites rather than auxin transport. Here, a new mode of action of flavonols to influence auxin homeostasis is revealed but yet the mechanism by which flavonols influence auxin conjugation and catabolism remains to be investigated. To uncover whether these secondary metabolites modulate other plant hormones, seedlings were grown in the presence of different hormones and root length was measured. This analysis demonstrates that *ugt89c1* mutation causes hypersensitivity of the roots to cytokinin treatment whereas no effects were noticed with other hormones. Plant hormones control many facets of plant growth and development. Both auxin and cytokinin have been shown to act either antagonistically or synergistically to control several developmental processes, such as the formation and maintenance of shoot and root meristem essential to build the entire plant body (Su et al., 2011). The hypersensitivity caused by the *ugt89c1* mutation toward cytokinin treatment might be due to the changed auxin homeostasis generated by the lack of 7-rhamnosylated flavonols. Alternatively, it is possible that 7-rhamnosylated flavonols are involved in the balance of the process catalyzing/degrading exogenous cytokinins because the spatial and temporal control of these processes is of key importance for proper growth (Kuroha et al., 2009; Miyawaki et al., 2004; Sakakibara, 2006; Werner et al., 2003). Unfortunately, quantification of cytokinin was not successful and therefore we could not determine whether the cytokinin metabolites are altered in *rol1-2* seedlings. Thus, it was of importance to investigate whether *ugt89c1* mutation affects the level of the other hormones. Quantification of different hormones such as ABA, salicylic acid (SA), jasmonic acid (JA) and the ethylene precursor, 1-aminocyclopropane-1-carboxylic

acid (ACC) in *rol1-2* seedlings compared to wild-type Columbia and *rol1-2 ugt89c1* double mutant compared to *rol1-2* single mutant revealed an increased level of these hormones in *rol1-2* mutant plants while *ugt89c1* mutation shows mitigation of the level of these hormones. Whether this is a result of hormonal crosstalk or individual effects of the change in flavonol glycosylation remains to be assessed. It will be of importance to further investigate this by crossing the *rol1-2* and *rol1-2 ugt89c1* mutants with different mutants defective either in the signaling pathway or the biosynthesis of hormones and quantify the level of the other hormones in these lines. Mutants to be used can be *abscisic acid deficient* mutant (*aba1* and *aba2*), a knock-out mutant defective in the jasmonic acid biosynthesis (*aos*), *ethylene insensitive* mutants (*ein1* and *etr1*) and *salicylic acid induction deficient* mutant (*sid2*). If the observed changes were due to hormonal crosstalk, changing one single hormone would be expected to have multiple effects on other hormones. It seems that there is a mutual interaction between hormones and flavonol glycosides. Several studies have revealed that plant growth regulators such as cytokinins can be relevant factors in inducing changes in plant secondary metabolism (Su et al., 2011). Furthermore, other studies have discussed the effects of cytokinins (Ali and Abbas, 2003; Angelova et al., 2001) as well as the jasmonic acid (Hendrawati et al., 2006; ISHIHARA et al., 2002) on the accumulation of flavonol glycosides. Therefore, it will be interesting to analyze the flavonol accumulation in cytokinin deficient mutants in *rol1-2* background versus *rol1-2* and therefore look at the root phenotype.

**7-*O*-rhamnosylated flavonols affect stomatal related processes, probably via modulating the level of abscisic acid**

The *rol1-2* mutant shows an increased level of abscisic acid compared to wild-type Columbia. The *ugt89c1* mutation leads to the attenuation of this effect. Thus, it was worth to check whether the *ugt89c1* mutation affects aspects of plant development regulated by abscisic acid such as aperture, length and density of stomata. Our data show that the stomatal length and density were significantly reduced by the *ugtc89c1* mutation compared to wild-type Columbia. Indeed, flavonols were shown to accumulate in guard cells (Watkins et al., 2014) and modification in the glycosylation profile of these compounds has been reported to affect stomatal density (Liu-Gitz et al., 2000; Ringli et al., 2008). The reduced stomatal aperture of *rol1-2* correlates with

the high level of abscisic acid found in these plants since that the phytohormone abscisic acid was shown to mediate stomatal closure in response to environmental stress (Cho et al., 2009; Hirayama and Shinozaki, 2007; Joshi-Saha et al., 2011; Zeevaart, 1980). Our data show that the *ugt89c1* mutation induces the stomatal opening compared to wild-type Columbia and in *rol1-2 ugt89c1* compared to *rol1-2* mutant plants. Moreover, flavonols are known to possess a radical scavenging ability (Pietta, 2000), which helps to keep the ROS level from approaching damaging levels within the cells. It is also known that $H_2O_2$ is involved in the abscisic acid signaling pathway as second messenger (Zhang et al., 2001;Murata et al., 2001); Kwak et al., 2003). Analysis of the NADPH oxidase *atrbohD/atrbohF* double mutant has led to understand further the implication of ROS on stomatal closure. These mutants show defective stomatal closure in response to abscisic acid (Kwak et al., 2003). The stomatal closure occurs via elevated ROS production generated by NADPH oxidase (Desikan et al., 2006). In guard cells, an increased level of abscisic acid leads to $H_2O_2$ production (Pei et al., 2000) via stimulation of NADPH or respiratory burst oxidase enzymes localized on the plasma membrane (Mustilli et al., 2002; Yoshida et al., 2002). Here we can speculate that the high abscisic acid accumulation in *rol1-2* might induce the increased ROS level and lead to stomatal closure in the *rol1-2* mutant. Whereas in the *ugt89c1* mutant, we can explain the increase in the stomatal opening by the reduced abscisic acid level which leads to low ROS production. Therefore, it is necessary to quantify the level of ROS in stomata of wild-type Columbia versus the *ugt89c1* mutant. Furthermore, it was reported that ROS decreases stomatal aperture throughout a $Ca^{2+}$ increase in the cytosol (McAinsh et al., 1996). It might be interesting to measure the $Ca^{2+}$ levels using for instance fluorescent indicator such as the Yellow chameleon (YC3.60) (Nagai et al., 2004) in the *ugt89c1* mutant versus wild-type Colombia and *rol1-2* versus *rol1-2 ugt89c1*.

**Flavonol-independent effect on plant growth induced by *ugt89c1***

The lack of 7-rhamnosylated flavonols in *rol1-2 ugt89c1* suggests an important role of these secondary metabolites in inducing the *rol1-2* shoot phenotype (Kuhn et al., 2016). We addressed the question whether a *ugt89c1* mutation has a flavonol-independent effect on plant growth and development in addition to rhamnosylating flavonols. Our results show that a mutation in *ugt89c1* induces a bulging phenotype

at the base of the root hair in the *rol1-2 fls1-3 ugt89c1* triple mutant i.e. in the absence of flavonols. The complementation of the triple mutant *rol1-2 fls1-3 ugt89c1* with a *UGT89C1:UGT89C1* construct successfully suppresses this phenotype. The bulging phenotype in the triple mutant reveals similarity with several mutant plants exhibiting alteration in root hair development such as *deformed root hairs1* (*der1*) that is defective in root hair development. The *DER1* locus codes for the ACTIN2 a major actin of the vegetative tissue (Ringli et al., 2002). The *root hair defective 1* (*rhd1*) mutants form short hairs with frequent bulges at the root hair base and is affected in a UDP-Glucose-4-Epimerase required for the galactosylation of xyloglucan (XG) and type II arabinogalactan during root hair development (Seifert et al., 2002). The *rho-related GTPase 2* (*rop2*) mutant shows impaired root hairs (Jones et al., 2002) and RHO-RELATED GTPASE (ROP) proteins regulate the organization of cortical microtubules and actin microfilaments (Fu et al., 2005). Furthermore, ROPs function in vesicle trafficking, actin organization and maintenance of ROS and $Ca^{2+}$ gradients (Baxter-Burrell et al., 2002; Carol et al., 2005; Fu et al., 2001; Hwang et al., 2005; Jones et al., 2002; Jones et al., 2007; Li et al., 1999; Molendijk et al., 2001; Wong et al., 2007). One major signal transducers for root hair growth are reactive oxygen species (ROS) (Mendrinna and Persson, 2015) and are regulated by the ROP proteins (Jones et al., 2007). It might be that UGT89C1 proteins are regulating ROS homeostasis; therefore it would be interesting to quantify ROS levels in wild-type Columbia versus *ugt89c1* mutants and *rol1-2* versus *rol1-2 ugt89c1*. An other major signal transducer for root hair growth is $Ca^{2+}$ (Mendrinna and Persson, 2015) since the $Ca^{2+}$ gradient is important for the root hair elongation (Bibikova et al., 1997). Also here, it would be interesting to measure the $Ca^{2+}$ levels in root hairs of *Arabidopsis* wild-type Columbia versus *ugt89c1* mutants and also investigate the cytosolic pH in these lines. Thus, the exact mechanism by which UGT89C1 affects the root hair development independently of flavonols remains to be uncovered. This could be via modifying intracellular processes or even cell wall formation.

**Flavonoid engineering**

During the two past decades, there was an increasing knowledge about flavonoid biosynthesis and flavonoids metabolites. Nowadays, there is rising demand for natural bioactive compounds as people express more interest about their health,

specifically in regards with health-promoting diets. The great characteristics of flavonoid compounds as anti-oxidant, anti-inflammatory, anti-microbial, and anti-cancer in plants, in human and animal health have made the biosynthetic pathways of flavonoids attractive targets for metabolic engineering. One application is the enhancement of the accumulation of these compounds, which is widely used in industry (Tanaka et al., 2008). Two major fields have benefited largely from flavonoids engineering. First, in horticulture, genetic modification of the flavonoid pathway has led to the establishment of ornamental plant species with new-pigmented flowers (Katsumoto et al., 2007; Zhai et al., 2014; Zuk et al., 2011). Second, improving plant defense by improving flavonoid production has been tested since flavonoids are known to act against pathogens in planta (Mierziak et al., 2014). Furthermore, it has become clearer that the composition of secondary metabolites enhances the quality and health potential of food and food products (Stobiecki et al., 2002). In addition to their important role in plant growth, development and responses to environmental stresses, these compounds have large impacts on agricultural productivity. Indeed, modification of flavonoid biosynthesis in crop plants such as maize was already tested in order to produce plants with increased nutritional value (Schijlen et al., 2004). Importantly, newly developed molecular tools such as the most widely used system Clustered Regularly Interspaced Short Palindromic Repeat-Associated protein CRISPR)/Cas (Bortesi and Fischer, 2015; Jinek et al., 2012) are available to genetically modify plants including numerous crops such as wheat, tomato, potato, maize and sugar beet (Song et al., 2016).

## 9.2 Outlook

The majority of enzymes and genes implicated in the flavonoid pathways have been widely described. However, distinct facets of flavonoid biology remain unknown. The findings presented here reveal that the flavonol biosynthetic enzyme FLAVONOL SYNTHASE 1 has additional activities, which are separable but not entirely independent. These activities, influencing growth process and inducing gene expression of genes coding for flavonoids biosynthetic enzymes, are of importance. They suggest a transcriptional regulatory effect of FLS1 and add another level of complexity to what was already known about the transcriptional control of this pathway. Yet, the mode of action of this protein to induce gene expression remains

to be elucidated. Our *in silico* analysis have shown the presence of potential DNA-bending domain in *FLAVONOL SYNTHASE 1*. Interestingly, a mutation produced in one of these DNA-binding domains, in *FLS1put2,* alters the ability of FLS1 to induce gene expression and inducing elongation of the root hair. It would be interesting to investigate the biological significance of these DNA-bending domain *in vitro* and *in vivo* using different methods such as *in vivo*-based ChIP-chip and ChIP-seq methods (Hanlon and Lieb, 2004; Horak and Snyder, 2002; Johnson et al., 2007; Lee et al., 2002; Park, 2009; Ren et al., 2000) and *in vitro* methods based on binding site enrichment (Zhao and Dixon, 2009; Zykovich et al., 2009). It will be also very important to uncover whether FLAVONOL SYNTHASE 1 interact directly with DNA or via a complex including flavonols i.e. the product of its own enzymatic activity. Here, two approaches can be tested to assess whether flavonols are required or not for the gene expression induced by FLS1; (i) preforming q-RT-PCR analysis to quantify gene expression by expressing nuclear *FLS1* gene in the *tt4-3* flavonoid-deficient mutant or (ii) using Dual Luciferase reporter system assay to investigate the induction of the gene expression directly by FLS1 in a transient expression system using the *tt4-3* flavonoid deficient mutant.

The use of *Arabidopsis thaliana* plants as a genetic tool obviously has helped in studying many aspects of plant secondary metabolism. The answer to the questions addressed in this chapter will be of great importance in order to achieve powerful engineering of the flavonoid pathway in plants. Data acquired using this species will help the extrapolation into other plants of agronomic and commercial interest.

# 10. Materials and Methods

## 10.1 Materials and Methods

### Plant material

The *rol1-2* and *fls1-3* lines used in this study are described in Kuhn et al. (2011) and Diet *et al.* (2006). The *ugt89c1* lines are described in Kuhn et al. (2016). For growth of plants in sterile conditions, seeds were surface sterilized with 1% sodium hypochlorite, 0.03% Triton X-100 and washed three times with sterile water, stratified 3-4 days at 4°C, and grown for 7 days on half-strength MS-medium containing 0.6% Phytagel (Sigma), 2% sucrose, 100 mg/l myo-inositol with a 16 h light / 8 h dark cycle at 22°C.

### DNA constructs, plant transformation and molecular markers

The *UGT89C1:UGT89C1* complementation construct in the binary vector *pART27* (Gleave, 1992) used in this study is described in Kuhn et al. (2016) and plants were transformed as described in Diet et al. (2006).

### Microscopic analysis of the root and root hair length

Microscopic analysis of the root length and root hair was performed using a binocular (Leica MZ125, Germany). For root length measurements, 30 seedlings for each line were grown in a vertical orientation for 8 days with increasing concentration of zeatin ($10^{-9}$ M, $10^{-8}$ M and $10^{-6}$ M). For the analysis of the root hairs, 20 seedlings were grown in a vertical orientation for 7 days in half-strength MS-medium.

### Stomatal aperture measurements

Stomatal aperture measurements were achieved as described in Pei et al., (1997). Tree independent abaxial epidermis strips were loaded onto glass slides with 300 µL of Arabidopsis leaf buffer consisting of 50 mM KCl, 10 mM MES, pH 6.1 (Melotto et al., 2006) and imaged by DIC microscopy using an Axioplan microscope (Zeiss, Jena, Germany). Stomatal aperture for each line was measured using the image-

processing software ImageJ and width / length ratio of stomata was determined using at least 200 stomata. Wide-open stomata have width / length values around 0.5,,while fully closed stomata will have a value of 0. A statistical analysis (pairwise T-test) was used to test for significance between different lines using a P-value <0.05.

**Hormone measurement**

Measuring the concentration of abscisic acid, jasmonic acid, salicylic acid and ethylene precursor (ACC) was performed in collaboration with Petre Dobrev/Eva Zazimalova, Prague, Czech Republic, following the extraction and purification method (Ivanov Dobrev and Kamínek, 2002). Followed by quantitation using LC-MS/MS (Dobrev and Vankova, 2012). Briefly, a sample (about 100 mg fresh weight) was homogenized in liquid nitrogen. The homogenate was supplied with 500 µl of cold extraction buffer (methanol/water/formic acid, 15/10/5, v/v/v, -20° C) and with a mixture of stable-isotope-labelled internal standards: $^{13}$C6-IAA (Cambridge Isotope Laboratories, MA, USA), $^{2}$H5-15N1-IAA-Asp, $^{2}$H5- 15N1-IAA-Glu, $^{2}$H2-OxIAA (OlChemIm, Olomouc, Czech Republic), 10 pmol each. After incubation for 30min at -20° C, the extract was centrifuged at 28000 x g (centrifuge Eppendorf 5430 R, Hamburg, Germany) and pellet was re-extracted once. Pooled supernatants were evaporated in vacuum concentrator (Alpha RVC, Christ, Osterode am Harz, Germany) and then, a sample residue was dissolved into 0.1 M formic acid and applied to mixed mode reversed phase–cation exchange SPE column (Oasis-MCX, Waters, MA, USA). Auxin and its metabolites were eluted with methanol. Elute was evaporated to dryness in vacuum concentrator and dissolved into 30 µl of 15 % acetonitrile. An aliquot was analysed on HPLC (Ultimate 3000, Dionex, CA, USA) coupled to hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems, MA, USA) set in selected reaction monitoring mode. Quantification of hormones was done using isotope dilution method with multilevel calibration curves ($r^2$>0.99). Data processing was carried out with Analyst 1.5 software (Applied Biosystems).

## Hypocotyl measurements

Seedlings were grown for 7 days in a vertical orientation in half strength MS-medium under unilateral white-light illumination (2 µmol $m^{-2}s^{-1}$) and with a 16 h light / 8 h dark cycle at 22°C. The hypocotyl length analysis was performed by measuring the distance from the collet of root hairs to the 'v' made by the cotyledon shoulders with ImageJ. To make accurate measurements using ImageJ, the program was first calibrated by drawing a line over a known distance on a picture of a ruler taken at the same magnification as the pictures of seedlings to be measured. Then, the average of at least 24 seedlings was calculated. A statistical analysis (pairwise t-test) was used to test for significance between different lines using a P-value <0.05. Error bars represent standard error.

## Measurement of hypocotyl curvature from vertical axis

10 day-old seedlings were grown vertically in half strength MS-medium under unilateral white-light illumination (2 µmol, $m^{-2}$ $s^{-1}$) and with a 16 h light / 8 h dark cycle at 22°C. Arabidopsis hypocotyl phototropism was performed as described by Gupta *et al.* (2012). Digital images of hypocotyl were captured after 10 days and the white-light-induced hypocotyl response was measured by calculating the angle of hypocotyl deviating away from the vertical axis. Hypocotyl curvatures were measured using the ImageJ program. The angle represents the average of two independent biological replicates having at least 26 seedlings and error bars represent standard error. The significance of differences between lines was confirmed by Student t-test.

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# 12 Curriculum vitae

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